**Ana Filipa Martins Oliveira**

Licenciada em Biologia

# Microglial clearance function: dependence on phenotypes

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

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Investigadora Coordenadora e
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

**Dezembro 2011**

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## ABSTRACT

Microglia are active sensors of the brain and respond promptly to even minor disturbance in their microenvironment. A feature of this response is the accumulation of these cells at the site of lesion. Neonatal jaundice is a common condition of the newborn and may determine injury to neurons and glial cells, such as microglia, when levels of unconjugated bilirubin (UCB) are excessive.

With the objective to evaluate whether microglia have a protective or deleterious role, we decided to assess, using the Boyden chamber, the chemotactic effect of free unbound UCB ($_f$UCB), as well as the migration ability of UCB-treated microglia in the absence or in the presence of chemotatic compounds, such as ATP and S100B. Also, we intended to evaluate the effect of glycoursodeoxycholic acid (GUDCA) as a modulator. To characterize our usual model of microglia isolation, phenotypic evaluation of cultures with different days *in vitro* (DIV) was performed by estimating cell morphology, nuclear factor-kappaB (NF-κB) activation and phagocytic ability.

We observed that $_f$UCB did not act as a chemotactic compound for microglia and that cells treated with UCB showed decreased migration ability. Co-incubation with GUDCA prevented this effect and enhanced microglia migration. However, reduced effects were observed in the presence of ATP and abolished when using S100B. Isolated microglia with 2 DIV showed features of activation, but presentedramified morphology of the “resting” state, less NF-κB activation and increased phagocytosis at 13 DIV.

Data indicate that microoglia exposure to UCB leads to a reduced migration ability and that co-incubation with GUDCA prevents this deleterious effect, resulting in an increased migration. Characterization of microglia phenotypes, along the time in culture, point to 13 DIV cells as the most suitable for studies intended to evaluate microglia reactivity to UCB, and probably to other stimuli.



Keywords: Unconjugated bilirubin; Microglia morphology; Cell migration; Phagocytosis; Nuclear factor-kappaB; Glycoursodeoxycholic acid.

## RESUMO

Amicroglia são células vigilantes activas, respondendo prontamente à mínima alteração do parênquima cerebral, acumulando-se no local de lesão. A icterícia neonatal, frequente no recém-nascido, pode causar dano nos neurónios e células gliais, como a microglia, quando as concentrações de bilirrubina não conjugada (BNC) são excessivas. Tendo por objectivo a avaliação do papel protector ou prejudicial da microglia, decidimos determinar, usando a câmara de Boyden, se a BNC livre, não ligada à albumina, exercia algum efeito quimiotáctico na microglia, bem como na capacidade de migração da microglia tratada com BNC, na presença e na ausência de agentes quimiotácticos, como o ATP e o S100B. Pretendeu-se ainda, avaliar se o ácido glico-ursodesoxicólico (AGUDC) exercia algum efeito modulador. Para caracterizar o fenótipo da microglia obtida no nosso modelo, procedeu-se à caracterização de culturas com diferentes dias *in vitro* (DIV), através da avaliação da morfologia celular, da activação do factor nuclear-kappaB (NF-κB), e da capacidade fagocítica.

Observámos que a BNC livre não exerce quimiotaxia e que a microglia tratada com BNC parece estar danificada e ter menos capacidade para migrar, efeito que foi invertido quando co-incubámos as células com AGUDC. Contudo, tal efeito foi reduzido na presença de ATP e não se verificou na presença de S100B. A microglia mostrou estar activada aos 2 DIV, mas apresentou uma morfologia ramificada, característica do estado vigilante, menor activação do NF-κB e aumento da fagocitose, quando avaliada aos 13 DIV.

Os resultados obtidos indicam que a exposição da microglia à BNC leva à perda da sua capacidade de migração e que a co-incubação com AGUDC, não só reverte como aumenta a migração. A caracterização dos fenótipos da microglia aponta para as culturas de 13 DIV como as mais adequadas para estudos que pretendam avaliar a reactividade da micróglia à BNC e provavelmente a outro estímulo.



Palavras-chave: Bilirubina não-conjugada; Morfologia da microglia; Migração celular; Fagocitose; Factor nuclear-kappaB; Ácido glico-ursodeoxicólico

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## ABBREVIATIONS

| | |
|---|---|
| **AD** | Alzheimer's disease |
| **Aβ** | Amyloid β |
| **BBB** | Blood-brain barrier |
| **BDNF** | Brain-derived neurotrophic factor |
| **CNS** | Central nervous system |
| **CR** | Complement receptor |
| **CSF** | Colony stimulating factor |
| **CY** | Cyanine |
| **DIV** | Days *in vitro* |
| **DMEM** | Dulbecco's Modified Eagle Medium |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **eNOS** | Endothelial nitric oxide synthase |
| **FBS** | Fetal bovine serum |
| **FcR** | Fc receptor |
| **GFAP** | Glial fibrillary acidic protein |
| **GUDCA** | Glycoursodeoxycholic acid |
| **HSA** | Human serum albumin |
| **Iba1** | Ionized calcium-binding adaptor molecule 1 |
| **IFN** | Interferon |
| **IL** | Interleukin |
| **LPS** | Lipopolysaccharide |
| **Mac-1** | Macrophage receptor 1 |
| **MAP-2** | Microtubule associated protein-2 |
| **MHC** | Major histocompatibility complex |
| **MMP** | Matrix metalloproteinase |
| **NF-κB** | Nuclear factor-kappaB |
| **NO** | Nitric oxide |
| **PAMP** | Pathogen-associated molecular pattern |
| **PBS** | Phosphate buffered saline |
| **PD** | Parkinson's disease |
| **PKC** | Protein kinase C |
| **PR** | Phosphatidylserine receptor |
| **PRR** | Pattern recognition receptor |
| **PS** | Phosphatidylserine |
| **PS1** | Presenilin 1 |
| **PVP** | Polyvinylpyrrolidone |
| **ROS** | Reactive oxygen species |
| **SR** | Scavenger receptor |

| | |
|---|---|
| **TAMs** | Tumor-associated macrophages |
| **TGF** | Transforming growth factor |
| **$T_H$** | **Helper** T cell |
| **TLR** | Toll-like receptor |
| **TNF** | Tumor necrosis factor |
| **TREM-2** | Triggering receptor expressed on myeloid cells-2 |
| **UCB** | Unconjugated bilirubin |

# I. INTRODUCTION

# I. INTRODUCTION

## 1. Microglia: introduction and overview

The survival and proper function of neurons is ensured by the large number of glial cells (Streit, 2002). Among this group of cells, a unique population resembling neural environment-adapted macrophages comprises upwards 12% of the non-neuronal brain cells and is designed by microglia (Aloisi, 2001; Ladeby *et al.*, 2005). These cells are ubiquitously distributed in non-overlapping territories throughout the central nervous system (CNS) including the spinal cord, but they vary in density, with the white matter generally containing fewer microglia than the grey matter (Soulet and Rivest, 2008; Ransohoff and Perry, 2009).

Since these cells derive from a mononuclear phagocyte lineage, they share several morphological features and functions with macrophages. Thus, microglia constitute the brain's immune system contradicting the initial idea of CNS as an immune privileged site (Kreutzberg, 1996; Streit, 2002). For this reason, microglia play an essential role in both physiological and pathological conditions (Polazzi and Monti, 2010), as it will be further discussed.

### 1.1. Microglia phenotypic diversity

For a long time, microglia phenotypes were solely divided in a resting and an activated state. In healthy normal CNS, microglia would be in a resting state presenting small cell soma with rod-shaped nuclei and numerous branched processes. In pathological conditions, microglia would turn into an activated state undergoing several morphological alterations, like the acquisition of an amoeboid shape with processes retraction, upregulation of cell surface markers and production of a plethora of bioactive mediators (Kim and de Vellis, 2005; Hanisch and Kettenmann, 2007). Cells in the resting state have been for a long time considered to be quiescent and inactive. Also, it was described that conversion to an activated state occurred nonspecifically irrespective of the injury, that is, in a stereotyped manner with a predetermined program of functions to execute (Davis *et al.*, 1994; Gehrmann *et al.*, 1995).

Recent reports, however, demonstrate that although microglial cell bodies are relatively fixed, their fine processes present an elevated motility, higher than that of astrocytes, allowing them to constantly palpate and monitor their microenvironment (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Formation of these processes occurs even in absence of any pathogenic stimulus suggesting that this formation may be associated with the integration of homeostatic signals throughout the CNS (Raivich, 2005; Carson *et al.*, 2007). Thus, these cells are highly active in their presumed resting state and the term "surveillant" has been advanced to replace this traditional definition (Ransohoff and Cardona, 2010). Furthermore, the activation process is adaptive so that microglia response is specific for each stimulus and brain region, and depending on the circumstances, this response may have neuroprotective or neurotoxic outcomes. (Carson *et al.*, 2007). For instance, expression of some surface molecules associated with immune function show brain regional variation (de Haas *et al.*, 2008).

Therefore, microglial activation is not a simple on-off switch and the heterogeneity appears to be an adaptation to support the diversity of neurons present in CNS (Hanisch and Kettenmann, 2007;

Silva *et al.*, 2010). In fact, microglial cells can detect microdamages in their neighborhood and play regenerative functions without undergoing a drastic transformation or initiating an inflammatory response. However, when stimuli are stronger or prolonged, microglial cells undergo more dramatic changes (Hanisch and Kettenmann, 2007).

Recently, it has been debated the concept of different states of activation of microglia accounting for the differing functional properties of these cells (David and Kroner, 2011). The idea emerged from research in non-CNS field in an attempt to perceive whether macrophages play a harmful or beneficial role after injury and which revealed important insights into macrophage polarization. Analogous to the T helper ($T_H$) 1/ $T_H2$ dichotomy of T cell polarization, macrophages can be polarized by the microenvironment to mount specific M1 or M2 functional programs (Porta *et al.*, 2009). Classical (M1) activation, induced by interferon (IFN)-γ, is characterized by a robust pro-inflammatory response required for the elimination of extracellular pathogens. On the other hand, interleukin (IL)-4 triggers an alternative (M2) activation important for the immune response to parasites as well as for tissue repair (David and Kroner, 2011; Saijo and Glass, 2011). Since macrophages are innate immune cells, their primary function is to act as phagocytes in response to pathogens. Nevertheless, they are capable of calling adaptive immune cells, despite the limited ability to process and present antigen to T cells, so that both arms of immune response act in concert to restore the homeostasis (Town *et al.*, 2005). Curiously, the populations of effector CD4 T cells intervening in the response are determined by macrophage polarization. For example, M1 cells release IL-12 that leads to activation of $T_H1$ which, in turn, release IFN- γ that induce long-lasting classical activation (Martinez *et al.*, 2009).

However, these simplified polarization states (M1 and M2) describe a complex process and are the extremes of a spectrum of functional states (Mantovani *et al.*, 2004). In fact, subgroups of M2, like M2a, M2b and M2c, have been used to further define macrophage polarization, since this general designation encompasses cells with dramatic differences in their biochemistry and physiology (Gordon, 2003; Mantovani *et al.*, 2007). Furthermore, it has also been proposed a distinct division of macrophage populations into three groups based on their homeostatic activities – host defense, wound healing and immune regulation (Mosser and Edwards, 2008).

The transposition of these concepts to microglia is quite simple in the case of classical activation and might also be applicable in the case of alternative activation. However, the definition of the steady-state in the case of microglia is more challenging and the determinants of this state might be very distinct from those imposed to macrophages. Moreover, it is not known if the deactivation of microglia results in a state functionally similar to the resting state. Notwithstanding, reports available are sufficient evidence to support the association between distinct phenotypes and pathology (Saijo and Glass, 2011)

Therefore, macrophages and microglia recognize signals from their microenvironment that induce specialized activation programs (Martinez *et al.*, 2009) leading to divergent effects in response to CNS injury (Kigerl *et al.*, 2009). Thus, depending on their differentiation status, microglia differently affects cellular function with either a deleterious or beneficial outcome in the repair (Miron *et al.*, 2011).

Hence, traditional definition of microglial phenotypes based on classic morphologic studies is now being replaced by a definition of functional states (Fig. I. 1). This new definition accounts for

differences in activation states undergone by microglia to achieve appropriated effector responses for each challenge to CNS (Ransohoff and Cardona, 2010). The recent discovery of microglial involvement in neurogenesis, postlesional and "synaptic stripping," underscores the existence of additional, functionally adapted microglial phenotypes (Graeber, 2010).

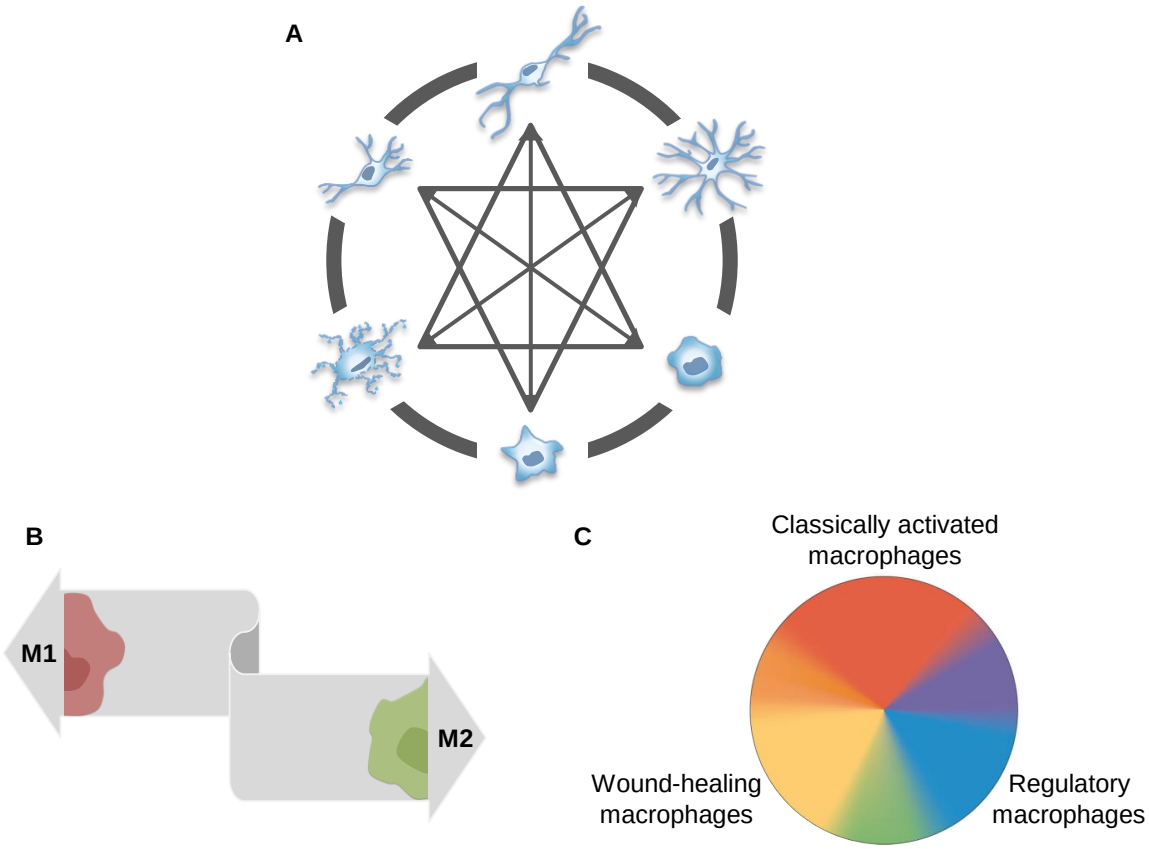


**Fig. I. 1: Microglial phenotypic diversity.**
**A.** Microglial cells show a great phenotypic heterogeneity and plasticity being capable of quickly adaption to achieve an appropriated effector response for each challenge to CNS. **B.** Recently, it was proposed the concept of different states of activation, ranging from "classical" activation (or M1) to "alternative" activation (or M2), which represent the extremes of a spectrum of functional states. **C.** Other authors extended the classification into three groups based on their homeostatic activities. They are arranged according to the three primary colors, with red designating classically activated macrophages, yellow designating wound-healing macrophages and blue designating regulatory macrophages. Secondary colors, such as green, purple and orange, represent cells that share properties from two of those groups.

Functional and phenotypic heterogeneity of these cells imply that no simple expression signature exist for microglia (Graeber and Streit, 2010). Indeed, in uninjured CNS, macrophages and microglia in different regions of the brain show differences in morphology and surface markers. This allows "resting" microglia to be distinguished from activated macrophages/microglia by their low CD45 expression, but in the injured CNS, this is no longer possible (David and Kroner, 2011). Therefore, along with a new definition, it becomes essencial to seek for new means of identifying this complexity of microglial phenotypes.

### 1.2. Role of microglia in critical periods of brain development

Distribution of microglia in the CNS varies with the different stages of brain development and is accompanied by the acquisition of different functions (Cuadros and Navascues, 1998). The roles of microglia during early stages of brain development, namely neonatal/postnatal period, and in aging brain are critical for the proper functioning of this organ and determine, in part, the possible occurrence of long-term neurological disabilities. Therefore, the next two topics will be intended to review these issues.

#### 1.2.1.Early brain development

The origin of microglia has been at the centre of debate since their first documentation by del Rio-Hortega (1932) according to whom microglia were derived from the invasion of mesenchymal pial elements during embryonic development. Currently, it is generally accepted by most authors that these cells derive from progenitors, at the yolk sac, that arrive to the developing CNS through the bloodstream, ventricles and meninges, at an early embryonic stage (Imai *et al.*, 1997; Chan *et al.*, 2007; Bilbo and Schwarz, 2009) (Fig. I. 2). In contrast,other authors still believe that microglia have a neuroectodermal origin arguing that neuroectodermal matrix cells are able to differentiate into microglia locally (Fedoroff, 1995).

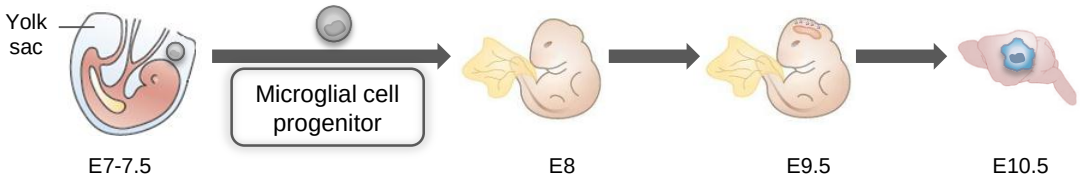


**Fig. I. 2. Origin of microglial cells.**
Microglial cells originate from primitive progenitors that arise in the yolk sac early during embriogenesis. They enter the embryo at the embryonic day (E) 8 and surround the neuroepithelium by E9.5. At E10.5, the earliest microglia are found in the brain. *Adapted from Ransohoff, 2011 and Saijo, 2011.*

There is a “developmental window” during which the microglial progenitors infiltrate into the CNS and that extends from embryonic day (E) 10 to E19 in rodents and from the latter half of the first trimester and throughout the early part of second trimester in humans (Rezaie and Male, 2002b; Rezaie and Male, 2002a). After entering the brain, these precursors originate microglial cells that still retain features of their progenitors presenting an amoeboid morphology and being highly motile, phagocytic and proliferative (Schlegelmilch *et al.*, 2011). They then migrate by tangential and radial migration and proliferate as amoeboid microglia becoming distributed throughout the nervous parenchyma (Marín-Teva *et al.*, 1998; Navascues *et al.*, 2000). First appearance of these macrophage-like microglia occurs about E14 in rat brain and continues to increase in density reaching a peak within the first postnatal week, between postnatal day (P) 4 and P8, with slightly variability depending on brain region (Wu *et al.*, 1992; Ling and Wong, 1993). During this time window, amoeboid microglia plays a central role in phagocyting cellular debris resulting from apoptosis of overproduced neurons (Kim and de Vellis, 2005; Schlegelmilch *et al.*, 2011). Also, even survival and neuronal death itself can be regulated in part by microglia through the release of growth factors and

cytokines that not only influence neurons but all the other surrounding cells (Kreutzberg, 1996; Hanisch, 2002). Indeed, cytokines are involved in many important processes in which microglia are committed like neurogenesis, synaptogenesis and gliogenesis (Santambrogio *et al.*, 2001; Nawa and Takei, 2006). Later in development, microglial cells also participate in neuritogenesis as well as in axonal growth and guidance of neurons (Streit, 2001) and it has also been suggested that they can promote vasculogenesis and angiogenesis (Pennell and Streit, 1997). Some of these processes extend from embryonic period throughout adolescence or even adulthood (Rice and Barone, 2000).

When microglia is mainly in an amoeboid/phagocytic state or present solely primitive ramification, they can respond promptly to infection or injury (Ling and Wong, 1993; Santos *et al.*, 2008). Indeed, several studies report cases of neonatal pathological conditions in which microglia is involved, like hypoxic-ischemic injuries (Tahraoui *et al.*, 2001; Kaur and Ling, 2009; Deng *et al.*, 2010) and excitotoxic brain damage (Dommergues *et al.*, 2003). In these cases, concomitant to the microglial activated phenotype, several surface markers are up-regulated, like major histocompatibility complex (MHC) class II and complement receptors (Greensmith and Navarrete, 1994; Ábrahám and Lázár, 2000). Also, inflammatory transcription machinery starts with nuclear factor-κappaB (NF-κB) activation (Nijboer *et al.*, 2008), leading to the release of mediators and modulators of inflammation that contribute to aggravate the pathological condition (Lai and Todd, 2006). Although this is the general outcome, some factors can have neuroprotective role following trauma (Ellis *et al.*, 2007; Nijboer *et al.*, 2008), which will be further discussed.

Pathologies in the neonatal period can have significant repercussions later in life, since it has been demonstrated that they can result in a phenomenon called “glial priming”. In this case cells become sensitized by an insult such that subsequent responses to future challenges are exacerbated (León-Chávez *et al.*, 2003; Perry *et al.*, 2003) and can lead to cognitive and behavioral impairments (Perry *et al.*, 2002; Nawa and Takei, 2006; Cooke and Abernethy, 2010). It is not without precedent that early development exposure to neurotoxic agents may determine latent effects on both morphological and behavioral endpoints which are manifest during the aging process (Barone *et al.*, 1995). Furthermore, it should be noted that the first postnatal weeks are particularly critical due to maximal brain growth rate, implying that any alteration will impact on neurogenesis ability and brain function in later life (Georg Kuhn and Blomgren, 2011). At about P15 in rodents (and from late second to early third trimester in humans), microglia reach their final destination in CNS parenchyma and undergo differentiation, a last major process that follows proliferation and migration. This sequence of events often referred to as “developmental” plasticity culminates with amoeboid microglial cells lastly turning into fully mature, ramified microglia (Bilbo and Schwarz, 2009; Tambuyzer *et al.*, 2009). These processes-bearing cells constitute the “resident” microglia of the adult brain.

### 1.2.2. Aging brain

The changes that occur in microglia with normal aging of the brain is an issue of great interest since it can provide us insights about neurodegenerative disorders to which aging is the major risk factor. Aging itself is not considered a disease but it appears to result in increased microglia activation, complement factors, inflammatory mediators and brain atrophy (Lucin and Wyss-Coray, 2009).

Microglia from aged brain shows amoeboid-like morphology, increased proliferation and antigen expression with up-regulation of MHC class II, as well as increase in inflammatory cytokines production (Perry *et al.*, 1993; Cagnin *et al.*, 2001). They also showed enhanced proliferation and resistance to a down-regulated phenotype, losing the sensibility to the anti-inflammatory cytokine transforming growth factor (TGF)-β1 (Rozovsky *et al.*, 1998). However, the chronic use of non-steroidal anti-inflammatory drugs appears to be effective in treating Alzheimer's disease (AD) by diminishing microglial activation (Mackenzie and Munoz, 1998). This is particularly true if nonsteroidal anti-inflammatory drugs are used at the earliest possible times as preventive instead as a recovering therapy (Varvel *et al.*, 2009)

Reactive microglial profile can lead to an exaggerated release of pro-inflammatory cytokines in case of activation of innate immune system that may lead to behavioral and cognitive impairments (Perry *et al.*, 2003; Cunningham *et al.*, 2005). What triggers increased microglial activation is still unclear but oxidative stress, resulting from increased reactive oxygen species (ROS) appear to have a determinant role (Floyd and Hensley, 2002; Lu *et al.*, 2004). In fact, gene-expression profile of aged human and mouse brains indicates an environment of inflammation and oxidative stress with reduced expression of genes related to synaptic function/transport, growth factors and trophic support (Lee *et al.*, 2000; Lu *et al.*, 2004). These global changes paint a bleak picture of the aged brain where neurons encounter increased challenges and receive reduced support.

Despite morphological and phenotypic changes indicating microglia activation, it has been proposed that microglia may undergo senescence, becoming dystrophic (Streit *et al.*, 2008). The morphological changes seen in this case are distinct from the ones that occur during microglia activation and include the loss of ramifications and even partial or complete fragmentation of cytoplasm, and nuclear condensation (Streit *et al.*, 2004b). This degeneration in normal aging brain could explain the high incidence of microglial apoptosis in AD brain reported by Lassmann *et al.* (1995). Dystrophy of microglia may reduce the secretion of neurotrophic factors and downregulate phagocytosis. Indeed, older rats reveal less clearance of myelin after a toxin-induced demyelination lesion (Zhao *et al.*, 2006). Furthermore, tau pathology was recently associated with dystrophic rather than activated microglia supporting the idea that it is the loss of microglial neuroprotection that contributes to the onset of sporadic AD (Streit *et al.*, 2009). Once dystrophic microglia accumulates in the aging human brain it is assumed that microglia undergo progressive deterioration.

In conclusion, it is probably the joint action of increased levels of inflammatory mediators and diminished levels of neurotrophic functions that lead to neuronal loss and inefficient clearance of toxic aggregates in neurodegenerative diseases (Fig. I. 3).

## 2. Functional roles of microglia

The most characteristic feature of microglial cells is their activation at a very early stage in response to even minor pathological changes in CNS (Kreutzberg, 1996). However, the functions of these cells extend beyond this ability, since they have important functional roles even in healthy brain (Nakajima and Kohsaka, 1993) (Fig. I. 4). Microglia play essential roles mainly during early brain development (as already discussed) and in the onset and progression of neurodegenerative diseases

where they can perform protective or toxic functions that will determine the brain functionality in the future (Perry *et al.*, 2010).

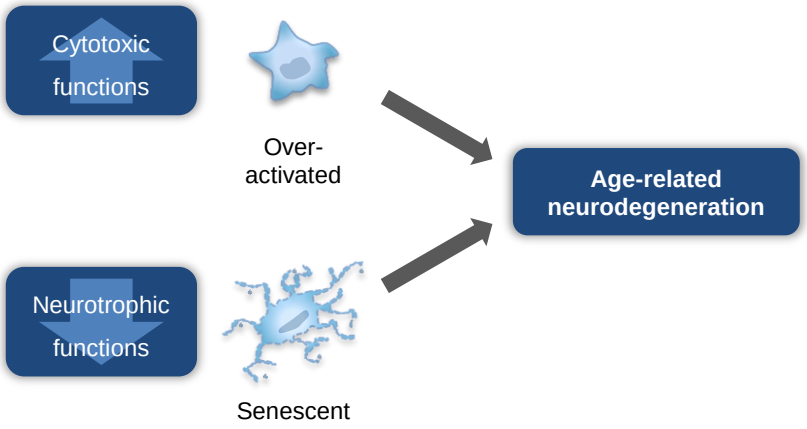


**Fig. I. 3. Microglia in the aged brain.**
During aging, microglial cells can become over-activated or senescent, resulting in increased pro-inflammatory response and decreased neuroprotective functions, respectively. Therefore, neurons encounter increased challenges and receive reduced support in the aged brain, accounting for neurodegeneration seen during age-related diseases.

The following sections will be dedicated to discuss the functional roles of microglia both in physiological and pathological brain.

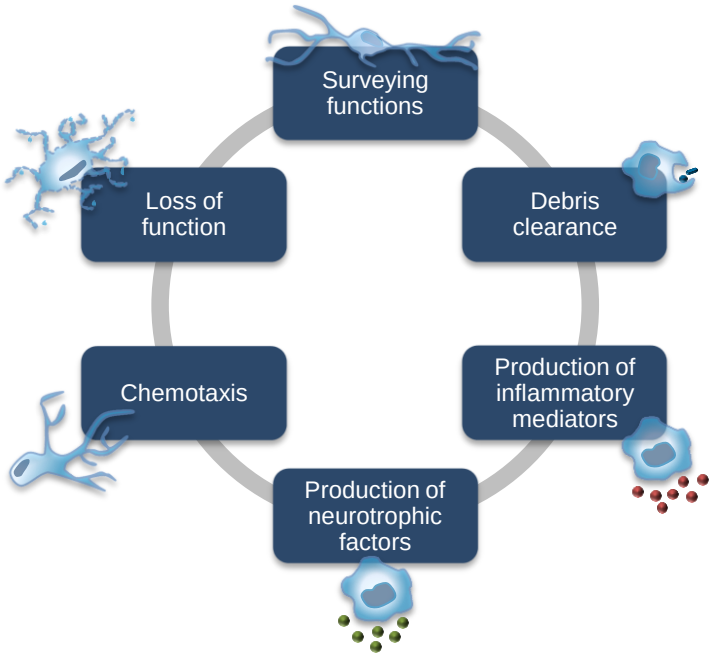


**Fig. I. 4. Functional roles of microglia.**
Resident microglia, when activated, adopt one of many diverse phenotypes and perform their functional roles during both healthy brain development and in response to pathological changes. *Adapted from Silva, 2010*.

### 2.1. Surveillance and repair

Microglia play a pivotal role in immune surveillance, host defense, and tissue repair, maintaining the homeostasis in the CNS (Liu *et al.*, 2001). During CNS development there is an intense remodeling in the brain since neurons must be generated in right quantity and location, and neural connections have to be properly established. Therefore, formation of mature neural circuits requires the elimination of inappropriate synaptic connections, a process termed as pruning. Recently, Stevens *et al*. (2007) demonstrated that the proteins C1q and C3b, both involved in the classical complement cascade, are required for this process as deficiencies in one or both proteins lead to defects in synapse elimination. This finding points to members of the complement cascade as new immune system players with also non-immune functions. The model is based in an early model from Jennings (1994) in which unwanted synapses are tagged by C3b and then phagocytosed by the resident microglia which express C3 receptor.

Since microglia appeareed to be involved in synaptic pruning, it is suggested that they might also contribute to plasticity after lesions (Cullheim and Thams, 2007). Indeed, an increased number of microglial cells is present both during development of CNS and in neurological diseases, reinforcing the idea of microglia involvement in synaptic remodeling in postnatal brain, but also in adult brain during pathological scenarios. Blinzinger and Kreutzberg (1968) showed that microglial cells caused the displacement of synaptic boutons from injured neurons, a process termed “synaptic stripping”. Similar observations have been made where activated microglia migrate and strip synapses during inflammatory processes and appear to have neuroprotective consequences (Trapp et al., 2007). In fact, there are three ways of synapse modification or elimination that are supposed to be mediated by microglia: proteolytic modification of the perisynaptic environment, remodeling of dendritic spine morphology, and phagocytic engulfment of dendritic spines, as well as of axon terminals (Tremblay and Majewska, 2011).

Microglia have also been pointed to induce neuronal apoptosis with the subsequent engulfment of cellular debris as demonstrated in cerebellar slice cultures where microglia showed to induce Purkinje cell neurons to undergo apoptosis (Marin-Teva *et al.*, 2004), and by a wide set of experiments evidencing that apoptotic cells are phagocytosed by microglia (Hume *et al.*, 1983; Perry *et al.*, 1985; Streit, 2001). This subject will be further discussed in section 2.2.3.

Microglia functions are not limited to differentiated cells but also in regulating neural stem/progenitor cells during both physiology and pathology. Walton and his colleagues (2006) demonstrated that conditioned meda from microglia were able to reconstitute neurogenesis and also showed that these cells may suffer alterations in their function with age, becoming more supportive of neuronal maturation in adult. Another study showed that in cases of injury to the CNS, activated microglia release soluble factors capable of directing migration of precursor cells towards sites of injury and also of influencing differentiation towards a neuronal phenotype (Aarum *et al.*, 2003). A role for microglia in promoting CNS lesion repair was further supported by studies with presenilin 1 (PS1) variants linked to the early-onset familial AD, in which proliferation and neuronal differentiation of progenitor cells are significantly impaired and associated with a decrease in the number of activated microglia. Furthermore, a different profile of secreted soluble factors was exhibited by microglia from

mice expressing the wild-type PS1 or PS1 variants suggesting a role for these factors in mediating adult hippocampal neurogenesis (Choi *et al.*, 2008).

Microglia also appear to exert functions that promote vascularization in the CNS. In an early study from Pennell and Streit (1997) it was observed that neural allografts were colonized by microglial cells before any blood vessels formation. Also, they continued to differentiate parallel to graft vascular development, and are often seen in close proximity to ingrowing vessels suggesting their participation in graft neovascularization. In fact, more recently, microglia were shown to differentiate from myeloid progenitors to facilitate vascularization in a model of ischemic retinopathy (Ritter *et al.*, 2006). The existence of tumor-associated macrophages (TAMs) with pro-tumoral functions raised the possibility that microglia, as brain resident macrophages, could have a role in angiogenesis (Allavena *et al.*, 2008). However, in this case, the outcome of this response might not be the one desired since TAMs produce factors that promote angiogenesis, remodel tissue and dampen the immune response to tumors (al-Sarireh and Eremin, 2000). Therefore, this particular case will be addressed later.

### 2.2. Role in innate and adaptive responses

Microglia reside in the healthy CNS as surveillant cells and are critically involved with both innate and adaptive immune system, regulating inflammation and cell damage in the brain (Chew *et al.*, 2006). They respond rapidly to changes in the CNS microenvironment and, depending on the condition, their response may have neuroprotective or neurotoxic outcomes (Ladeby *et al.*, 2005), as already mentioned.

Activation of microglia involves several features including cell morphological changes, upregulation of several cell surface markers, production of inflammatory mediators, as well as the ability to present antigens and to perform phagocytosis (Hanisch and Kettenmann, 2007). These features do not define one single microglial phenotype per every challenge to CNS, as aforementioned. Indeed, Town *et al.* (2005) consider that exists a continuum of microglial activation, with phagocytic response (innate activation) at one end and antigen presenting cell function (adaptive activation) at the other. Therefore, the nature and duration of stimuli influences the pathways of gene expression and the phenotypical changes that will occur afterwards, reinforcing the idea of plasticity in microglial response (Ransohoff and Perry, 2009; Parkhurst and Gan, 2010).

#### 2.2.1. Signals triggering activation

Transformation of microglia occurs following infectious diseases, inflammation, trauma, ischemia, brain tumors and neurodegeneration (Kreutzberg, 1996). These cells are under strict control of the neurochemical environment and its effects are site-specific, accounting for differences in the degree of microglia activation and inflammatory reactions in different CNS regions (Aloisi, 2001; Hanisch, 2002).

The first signal leading to the activation is the separationof microglia from their microenvironment inhibitory inputs that maintain them in a steady-state (Ransohoff and Cardona, 2010). In healthy CNS, microglia are constitutively restrained by inhibitory influences mainly produced by neurons (Hanisch, 2002) (Fig. I. 3). One of those signals is mediated through interaction of the fractalkine ($CX_3CL1$), released by neurons, with its receptor $CX_3CR1$ expressed on microglia. Ablation of the receptor

deregulates microglia responses resulting in neuronal death *in vivo*, as observed in several models of CNS insult (Cardona *et al.*, 2006; Ré and Przedborski, 2006). Interestingly, this function depend on cell-to-cell contact but this membrane-tethered chemokine can be cleaved and act as a soluble molecule with chemoattractive activity (Chapman *et al.*, 2000), an issue to be discussed in the next section. Interaction of the myeloid restricted molecule CD200R with its widely expressed ligand CD200 is also determinant to block microglia activation (Taylor *et al.*, 2011). Another example of an inhibitory signal derives from endogenous ligands of neurons that act on triggering receptor expression by myeloid cells-2 (TREM2), promoting phagocytosis and retarding inflammation (Hsieh *et al.*, 2009). When neurons are damaged, the communication through these "calming signals" is disrupted leading to microglia activation with the consequent increase of inflammatory response and neurotoxicity (Cardona *et al.*, 2006; Ré and Przedborski, 2006).

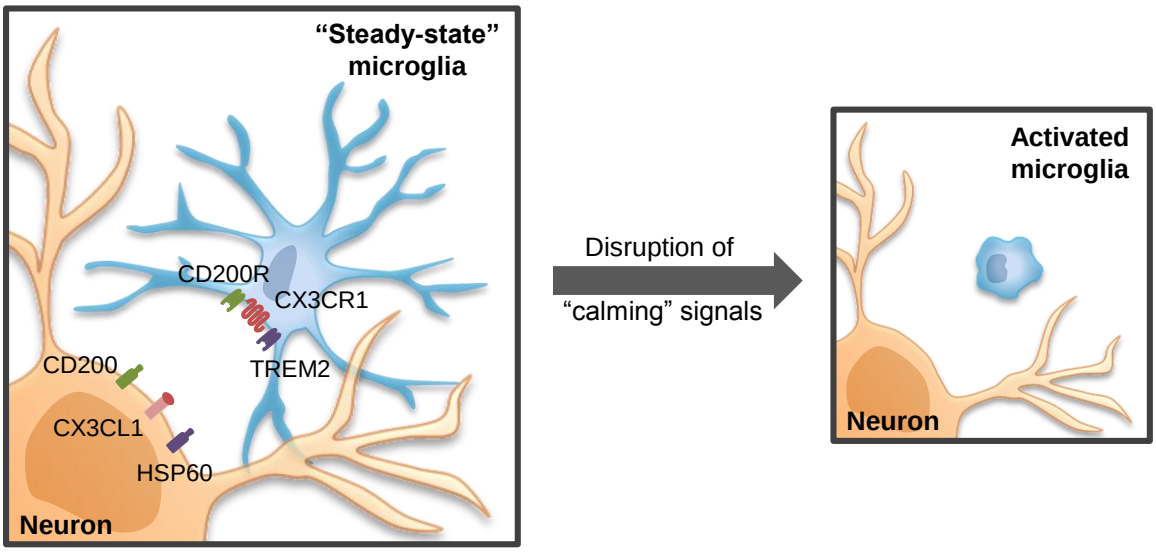


**Fig. I. 5. Steady-state microglia and homeostasis in the central nervous system (CNS).**
Under steady-state conditions, microglia exhibit an extensively ramified morphology and a resting phenotype. This phenotype is maintained in part through neuron-derived signals, including CX3CL1 and CD200, which act through corresponding receptors expressed by microglia. Also, functional triggering receptor expressed by myeloid cells-2 (TREM2) is essential to prevent excessive innate and adaptive immune responses. Disruption of these calming signals leads to microglia activation with a switch to a characteristic amoeboid morphology.

Microglia can also detect some factors that can lead to their activation. For example, serum constituents crossing the disrupted blood-brain barrier (BBB) (Ransohoff and Perry, 2009), altered synaptic activity leading to variations in neurotransmitters availability such as glutamate (Taylor *et al.*, 2003) and ATP (Davalos *et al.*, 2005), thus resulting in microglia activation. In the case of purine ATP that is released by damaged neurons, it acts on microglia to mediate processes extension (Gyoneva *et al.*, 2009) and the function of microglia as phagocytes (Koizumi *et al.*, 2007). Plasma fibrinogen can also bind to an engaged macrophage receptor 1 (Mac-1) /complement receptor (CR) 3 on activated microglia leading to increased phagocytic activity. Several cytokines and colony-stimulating factors (CSFs) also induce proliferation or phenotypic and morphological changes consistent with microglial activation (Kreutzberg, 1996).

In addition to endogenous signals, non-self components like bacterial and viral molecular patterns, recognized by toll-like receptors (TLRs) expressed on microglia, also lead to activation of these cells. Lipopolysaccharide (LPS), an Gram-negative bacterial endotoxin acting on TLR4, is widely used as an experimental activator since by changing microglia morphology and inducing the production of harmful factors. Indeed, LPS leads to microglial production of nitric oxide (NO), tumor necrosis factor (TNF)-α and IL-1β through the mediation of conventional protein kinase C ((Nakajima *et al.*, 2003).

### 2.2.2.Motility

Microglial cells are able to sense extracellular directional cues and to respond with asymmetric changes in cell morphology and motility.

In the healthy brain, "resting" microglia display a baseline motility characterized by the extension and retraction of their cellular processes without cell body movement (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005) but, after CNS injury, they can migrate to rapidly populate the site of injury (Morgese *et al.*, 1983). This movement of microglial cells leading to their accumulation at the site of lesion (a process called homing) is essential for the cellular immune responses of microglia and is triggered by "find-me" signals released by the damaged tissue (Azuma *et al.*, 2001; Kurpius *et al.*, 2007). Movement of microglia can be the result of a random, non-vectorial motility (chemokinesis), or a directed migration that depends from a chemical gradient to organize the movement (chemotaxis), or both (Devreotes and Janetopoulos, 2003; Miller and Stella, 2009).

Cell migration can be triggered by a diverse array of chemoattractants. Consistent with their role in the immune response in the CNS, microglia express an array of receptor for chemokines which are involved in the intercellular communication in brain and contribute to intracerebral recruitment of immune cells (Aloisi, 2001; Hanisch, 2002; Kim and de Vellis, 2005). Fractalkine, as mentioned in the previous section, is a chemokine that can exist in two different forms, membrane-anchored or as a soluble glycoprotein (Harrison *et al.*, 1998). Under normal conditions, $CX_3CL1$ is constitutively associated to the neuronal membrane, stimulating higher intracellular calcium mobilization in responding cells. This process is apparently needed for adhesion properties.However, after an excitotoxic stimulus, the chemokine undergoes a cleavage by matrix metalloproteinases (MMPs) triggered by the NMDA (N-methyl-D-aspartate) receptor pathway (Chapman *et al.*, 2000). The soluble glycoprotein can then interact with its receptor expressed on microglia inducing their chemotaxis (Harrison *et al.*, 1998). Thus, the release of this molecule is an early event in the inflammatory response leading to neuronal death after an excitotoxic injury (Chapman *et al.*, 2000). An intriguing aspect of the role played by $CX_3CL1$-$CX_3CR1$ pair in this inflammatory response is the fact of having different consequences under different pathologic conditions. While several disease models showed that $CX_3CR1$ deficiency deregulates microglial responses resulting in neurotoxicity *in vivo,* in mouse cerebral ischemia it is associated with better general recovery and reduced inflammation (Denes *et al.*, 2008). Moreover, microglial migration is also induced, among others, by the lymphoid chemokine CCL21, which involves chloride channels (Rappert *et al.*, 2002), and by secondary lymphoid-tissue (Biber *et al.*, 2001), both expressed by damaged neurons and acting on CXCR3 receptor expressed by microglia.

Microglial cells also migrate towards neurotrophic factors such as the epidermal growth factor (Nolte *et al.*, 1997) and nerve growth factor (De Simone *et al.*, 2007), corroborating the role of microglia in brain development. Pathological protein aggregates, such as amyloid-β (Aβ), have also been shown to induce microglial migration (Rogers and Lue; Gyoneva *et al.*, 2009).

Purines that are released from injured tissue in higher levels into the extracellular space by neurotransmission, lead to microglia chemotaxis (Parkhurst and Gan, 2010) (Fig. I. 6). Indeed, the baseline motility of microglial processes in the intact brain is modulated by some of the ATP signaling mechanisms mediating injury-induced microglial responses (Davalos *et al.*, 2005). Until now, process extension and migration is known to involve chloride channels (Hines *et al.*, 2009), ATP G protein-coupled purinergic receptors (P2YR) (Honda *et al.*, 2001; Haynes *et al.*, 2006), outward potassium currents (Wu *et al.*, 2007), astrocytic connexin hemichannels (Suadicani *et al.*, 2006), integrin β1 (Ohsawa *et al.*, 2010), signaling through phosphoinositide 3-kinase, Akt (Irino *et al.*, 2008; Franke *et al.*, 2009) and non-transcriptional activation of matrix metalloproteinase-9 (Choi *et al.*, 2010). In fact, microglial cells from mice lacking $P2Y_{12}R$ receptor are unable to polarize, migrate or extend processes towards nucleotides, either *in vitro* or *in vivo* (Haynes et al., 2006). Besides P2YR, ATP acts on ionotropic P2X receptors (P2XR) and $P2X_4R$ which are also involved in microglial chemotaxis as demonstrated by Ohsawa and his colleagues (2007). Moreover, the $P2X_7R$ mediate ATP release, essential for amplification of calcium signal transmission within the astrocytic network (Suadicani *et al.*, 2006). Furthermore, microglial cells themselves can release ATP so that a positive feedback mechanism may exist to perpetuate migration. Results from *in vitro* assays showed that ATP, as well as ADP, induce chemotaxis and membrane ruffling, a characteristic structure of migrating cells (Honda *et al.*, 2001). To notice that ATP also stimulates chemokinesis, which is independent of a chemical gradient (Miller and Stella, 2009). To add that ATP concentration released from injured cells may not be enough to generate a rapid microglial response in all brain pathologies (Davalos *et al.*, 2005). Intriguingly, Duan and his co-workers (2009), and more recently Samuels and his colleagues (2010), claim that nitric oxide (NO) is the true responsible for directing the movement of microglia after nerve injury in leech. According to these studies, ATP released by damaged cells activates microglia and induces their initial movement, whereas NO directs migration of the cells to CNS lesions. Indeed, the activity of the constitutive endothelial nitric oxide synthase (eNOS) is increased even before microglia accumulate, and persists during later stages of this accumulation (Chen *et al.*, 2000). In mammals, glial calcium waves, triggered by injury, lead to the release of ATP (Scemes *et al.*, 2007) and NO generation (Li *et al.*, 2003), thus indicatingthat theymay work in concert to induce microglia chemotaxis. In fact, NO mediates microglial response to acute spinal cord injury *in vivo*, but this process is under the control of ATP (Dibaj *et al.*, 2010). Nucleotide uridine diphosphate (UDP) is released by dying neurons and recognized by $P2Y_6$ receptor ($P2Y_6R$), specifically expressed on microglia. However, in this case, UDP cause $P2Y_6$ R-dependent phagocytosis *in vivo* and *in vitro*. Interestingly, ATP is not able to efficiently activate this receptor suggesting that microglia is first attracted by this “find-me” signal and subsequently recognizes UDP as an “eat-me” signal leading to the engulfment of the target (Inoue, 2007; Koizumi *et al.*, 2007).

Another curiousl aspect of this purinergic signaling is its involvement in the retraction of microglia processes. Indeed, a switch of the chemotactic response to ATP occurs in activated microglial cells, such as the one observed in LPS-pretreated microglia (Gyoneva *et al.*, 2009). This chemorepulsion away from ATP is a consequence of upregulation of a G protein-coupled adenosine receptor (AR), $A_{2A}R$, which is activated by adenosine resulting from rapid ATP breakdown by nucleotidases (Orr *et al.*, 2009). Also, simultaneously, there is the downregulation of $P2Y_{12}R$ as a result of TLR activation by LPS (Gyoneva *et al.*, 2009). Therefore, microglia extend their processes and migrate towards the local of lesion where they envelop the area and adopt an amoeboid morphology as the one seen during brain neuroinflammation and neurodegeneration.

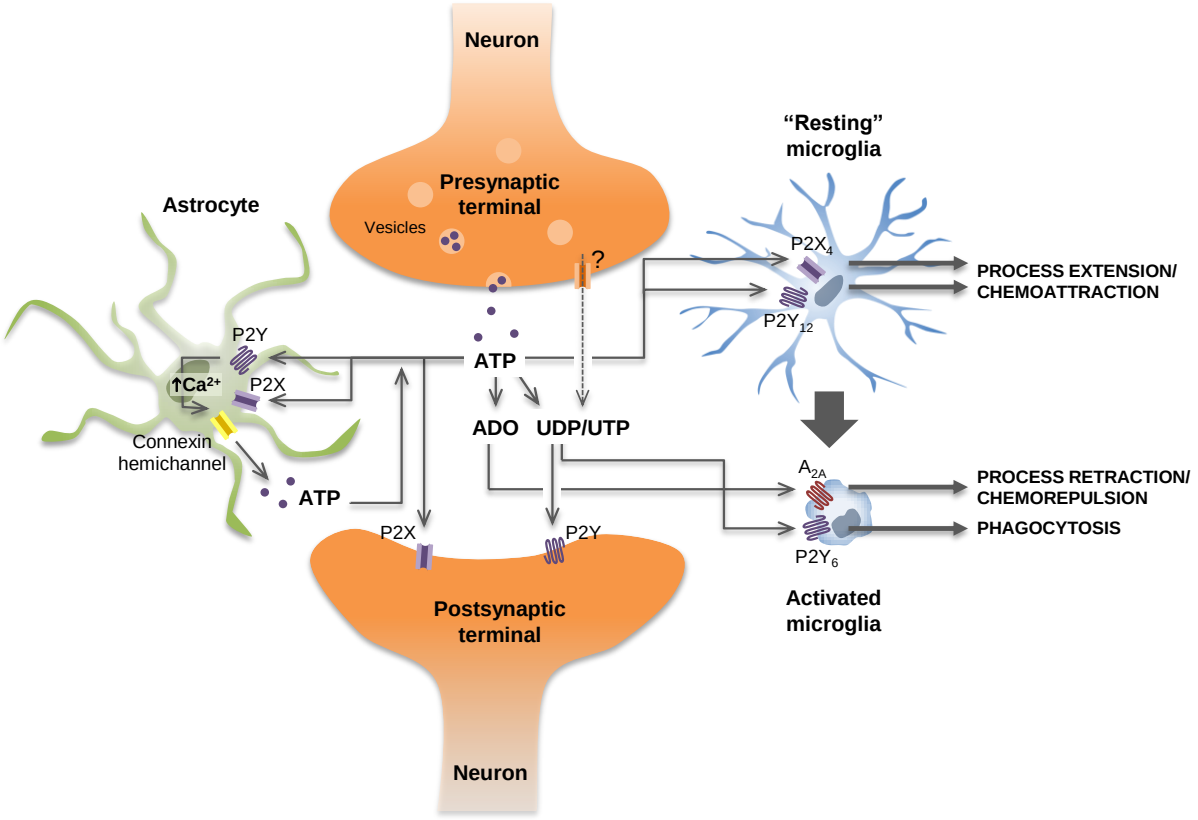


**Fig. I. 6. Purinergic signaling involved in microglia motility.**
Presynaptic neuron release ATP as a cotransmitter, by exocytosis. The released ATP acts postsynaptically on P2X and P2Y receptors activated by ADP, UTP and UDP, as well as ATP. ATP is also released from astrocytes (and probably from microglia, as well) to participate in glial–neuron interactions. Both P2X and P2Y receptor subtypes are expressed by astrocytes. Activation of P2Y receptors on astrocytes triggers a calcium (Ca2+) wave inducing the release of ATP through connexin hemichannels. P2X4 and P2Y12 receptors expressed on resting microglia mediate migration, whereas P2Y6 receptors that are expressed on the activated amoeboid microglia mediate phagocytosis of debris at the site of damage. Also, activated microglia express A2A receptors that are activated by adenosine (ADO) resulting from ATP breakdown by ectonucleotidases. Activation of such receptors induces chemorepulsion of microglial cells. Not depicted is the activation of potassium and chloride channels as well as signaling through phosphoinositide 3-kinase and Akt resulting from activation of P2Y12 receptors.

Another compound with chemoattractive function that was recently described is S100B. This protein is abundantly expressed in astrocytes but also in microglia and is released during the course of acute and chronic brain disorder (Ellis *et al.*, 2007). Once released, S100B exerts autocrine and

paracrine effects mostly by engaging RAGE (receptor for advanced glycation end products) on responsive cells. In low concentrations this protein might exert trophic functions, while in high concentrations, like those shown to be present in the extracellular milieu in case of brain damage, has pro-inflammatory effects leading to activation of microglia, enhancing migration and secretion of pro-inflammatory mediators (Bianchi *et al.*, 2011). For these reasons, S100B has been suggested to play a role in the pathophysiology of neurodegenerative and inflammatory brain diseases (Sorci *et al.*, 2010).

The signaling pathways involved in migration are diverse, as described above, and control different types of primary microglial cells movement (Miller and Stella, 2009).

### 2.2.3.Phagocytosis

Cells can take up macromolecules and particles from the surrounding medium in a process termed endocytosis. This is essential for ingestion and uptake of extracellular nutrients, for antigen presentation, and for removal of microbial pathogens. During endocytosis cells absorb the material or organisms from the extracellular space by engulfing them with their plasma membrane which then buds off inside the cell to form a vesicle containing the ingested material. Three principal mechanisms of endocytosis have been described. One of the best-characterized endocytic processes is receptor-mediated endocytosis via clathrin-coated pits. In this case cells internalize molecules by the inward budding of plasma membrane vesicles containing proteins with receptor sites specific for the compounds. The term pinocytosis refers to the ingestion of dissolved material or single molecules from the extracellular space. During this process the cytoplasmic membrane invaginates and pinches off small pinocytic vesicles which are then transferred to the cytosol. Phagocytosis is the third form of endocytosis involving the vesicular internalization of solid particles, such as microbial pathogens or apoptotic cell debris. Binding of the particle to receptors on the surface of the phagocytic cells stimulates the extension of pseudopodia, which eventually surround the particle, and fuse to form a large intracellular vesicle called phagossome. Once entered into the cell, the phagosome fuses with the lysosome, producing phagolysosomes in which the ingested material is digested (Silverstein *et al.*, 1977; Mukherjee *et al.*, 1997) .

Phagocytosis is a major function of activated microglia. This function is critical for the uptake and degradation of infectious agents and senescent cells, and it participates in development, tissue remodeling, immune response, and inflammation (Chew *et al.*, 2006). In general, microglial phagocytosis can be divided into two distinct responses: phagocytosis of pathogens and stimulation of TLRs inducing a pro-inflammatory cascade (Fig. I. 7A) and clearance of apoptotic cell membranes and recognition of phosphatidylserine (PS) residues inducing an anti-inflammatory response (Fig. I. 7B).

Microglia, as other phagocytic cells, have the capacity to discriminate a large number of potential pathogens from self antigenes, utilizing a restricted number of phagocytic receptors. This ability derivesfrom the capacity that these receptors have to recognize conserved motifs on pathogens, not found in higher eukaryotes, which by having essential roles in the biology of the invading agents, are not subjected to high mutation rates. These receptors are collectively termed pattern recognition receptors (PRRs), as they recognize specific pathogen-associated molecular patterns (PAMPs)

(Janeway, 1992; Aderem and Underhill, 1999). The major PRRs include the Fc receptors (FcRs) and the complement receptors (CRs) which recognize immunoglobulins and complement proteins coating the particles, acting as opsonins. Besides these, other receptors are involved in particle uptake, such as lectins, like mannose receptor that recognize mannans, other non-complement-receptor integrins and scavenger receptors (SR) that recognize surface components on bacteria including LPS(Aderem and Underhill, 1999; Underhill and Ozinsky, 2002). Multiple receptors are simultaneously engaged to mediate internalization, activate microbial killing, and induce the production of inflammatory cytokines and chemokines. Collectively, these cellular processes constitute the phagocyte response. Certain phagocytic receptors such as FcRs trigger inflammatory responses directly, whereas others, such as complement receptors, often do not stimulate inflammatory responses (Ravetch and Clynes, 1998). In many cases, these responses are regulated by additional receptors (that are not themselves phagocytic), such as TLRs, which are widely expressed on microglia. During phagocytosis, TLRs are recruited to the phagosomes, although recruitment does not require receptors activation (Underhill and Ozinsky, 2002). Nevertheless, they can sample the contents to determine the nature of the pathogen and participate in the formulation of an inflammatory response appropriate for defense (Aderem and Ulevitch, 2000). Also, activation of TLR signaling pathway by bacteria regulate internalization and phagosome maturation (Blander and Medzhitov, 2004). Concomitantly, TLRs activate NF-κB (Kawai and Akira, 2007) which can transactivate promoters of genes involved in immune and inflammatory responses (Baeuerle, 1991). Indeed, TLR4 can mediate LPS-induced macrophage activations of IL-1β and IL-6 gene expression, chemotaxis, phagocytosis, and oxidative ability (Wu *et al.*, 2009).

Microglial cells also play an important role in the recognition and clearance of apoptotic cells. Removal of apoptotic cells usually involves three central elements. First, the attraction of phagocytes via soluble "find-me" signals released signals by damaged cells, an issue already discussed in previous section. Second, recognition and phagocytosis via cell surface-presenting "eat-me" signals and finally, suppression or initiation of inflammatory responses depending on additional innate immune stimuli (Napoli and Neumann, 2009).

The best studied “eat-me” signal is PS, which translocates from the inner to the outer leaflet of plasma membrane at the early stage of apoptosis. Interestingly, it was recently demonstrated that viable cells can also express PS without leading to their phagocytosis indicating that the exposure of these residues alone is not sufficient to be recognized by microglia as an “eat-me” signal (Segawa *et al.*, 2011). The exposed PS on apoptotic cell is recognized by several phagocyte receptors including a presumptive PS receptor (PSR) (Savill *et al.*, 1993), even though experimental demonstration of such a receptor has been quite controversial (Williamson and Schlegel, 2002). Recently, several groups have identified receptors that both directly recognize PS and induce phagocytosis of apoptotic cells. These receptors include the brain-specific angiogenesis factor 1 (Park *et al.*, 2007a), the T-cell immunoglobulin domain and mucin domain 4 (Miyanishi *et al.*, 2007; DeKruyff *et al.*, 2010; Freeman *et al.*, 2010), and stabilin-2 (Park *et al.*, 2007b).

Although the mechanisms of recognition of apoptotic cells have not been completely resolved, several other molecules appear to be involved, as well. These include the milk fat globule factor-E8 which act as soluble bridging protein that binds to both the signal on the apoptotic cell and the

receptor on the phagocyte, thus acting as a collectin or opsonin (Fuller and Van Eldik, 2008). The arrest-specific gene 6 helps phagocytic cells in recognizing surface PS expression and facilitates the clearance of PS-expressing cells (Ishimoto *et al.*, 2000). SRs, such as CD36, appear to recognize membranes of apoptotic cells which are negatively charged (Husemann *et al.*, 2002) and CRs expressed on microglia recognize the complement protein C1q, which is able to bind to PS and act as a bridging molecule in apoptotic cell recognition during early stages of apoptosis (Paidassi *et al.*, 2008). Indeed, both receptors mediate myelin phagocytosis by microglia even though CR3 has a dominant role (Reichert and Rotshenker, 2003; Rotshenker, 2003). TREM2 interacts with ligands on apoptotic neurons, stimulating their removal and counter-regulating pro-inflammatory signals to allow repair (Neumann and Takahashi, 2007; Hsieh *et al.*, 2009).

Suppression of inflammation during removal of apoptotic cells involves both direct inhibition of pro-inflammatory cytokine production and release of anti-inflammatory factors, which all contribute to the resolution of inflammation. For instance, in acute multiple sclerosis lesions, removal of myelin debris by microglia leads to suppression of microglial inflammatory activity with downregulation of inflammatory cytokines and chemokines production (Liu *et al.*, 2006). Also, macrophages ingesting apoptotic cells showed increased production of TGF-β1, prostaglandin E2 (PGE), platelet-activating factor (Fadok *et al.*, 1998) and IL-10 (Zhang *et al.*, 2010). Interestingly, macrophages engulfing apoptotic cells release adenosine which triggers $A_{2A}R$ that mediates suppression of inflammation (Köröskényi *et al.*, 2011). Moreover, a mechanism dependent on the chemokine receptor CCR2, a major chemokine receptor for microglia- and blood-derived monocytes, is involved in the recruitment of cells from the bone marrow to limit Aβ plaque load by phagocytosis (Napoli and Neumann, 2009). Also in AD, loss of neuron-microglial fractalkine signaling ameliorates the condition that leads to reduced deposition of Aβ, by mediating an altered activation and phagocytic ability of these cells (Lee *et al.*, 2010). This demonstrates the intimate relation of phagocytosis and chemotaxis properties of microglia since, as already discussed, adenosine plays an important role in mediating the retraction of microglial processes and acquisition of an amoeboid morphology essential to perform their effector functions, such as phagocytosis. Indeed, both processes are actin polymerization-dependent since because cytoskeleton reorganization is required for their initiation (Peracino *et al.*, 1998).

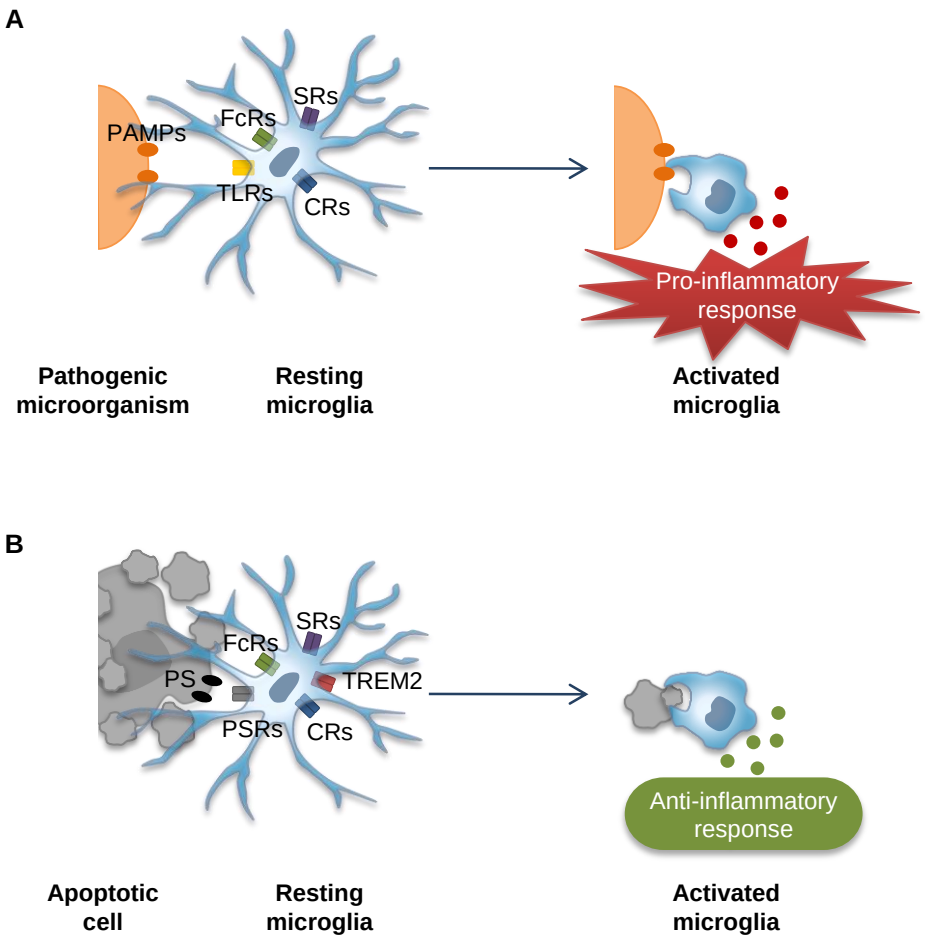


**Fig. I. 7. Phagocytic receptors of microglia.**
Microglia recognize specific structural patterns (PAMPs) of most microbial pathogens via their TLRs, complement receptors (CRs), Fc Receptors (FcRs) or scavanger receptors (SRs), leading to a pro-inflammatory response. B. Microglia recognize apoptotic cells through phosphatidylserine residues (PS) expressed on the membrane of the death cells. These residues are recognized by PS receptors, like Tim4, which are supported via additional phagocytic receptors including TREM2. Phagocytosis of apoptotic cells induces the release of anti-inflammatory cytokines *Adapted from Neumann, 2009 and Napoli, 2009.*

In conclusion, phagocytosis is a cell response with important functions in the course of an immune response, but also during tissue remodeling and wound healing. However, it is not a cell response that occurs as an isolated event, and a phagocytic stimulus triggers, besides associated cell responses of destructive nature, an immuno-modulatory cell response as well.

### 2.2.4.Neuroprotection *vs.* neurodegeneration: production of mediators

Microglial cells are the source of an array of mediators that may either have a neurotoxic or neuprotective effect (Fig. I. 8). During brain development and in physiological conditions, microglia contributes to growth, functional maintenance and phenotypic development of neuronal cells, as well as to proliferation and growth of glial cells by producing neurotrophic factors, such as the glial cell line-derived neurotrophic factor, basic fibroblast growth factor, brain-derived neurotrophic factor (BDNF), nerve growth factor and neurotrophins (Lindvall *et al.*, 1994; Nakajima *et al.*, 2001a).

Activation of microglia upon CNS injury has been considered as a detrimental event. This idea came from considering the inflammatory and cytotoxic phenotype acquired by LPS-stimulated microglia in cell-cultures as typical of activation (Schwartz *et al.*, 2006; Hanisch and Kettenmann, 2007). However, recent studies suggest that under pathological conditions, microglia exert neuroprotective functions through the production of neurotrophic molecules and by clearance of cell debris (Kitamura *et al.*, 2009). In fact, microglial activation by an acute CNS injury intends to ameliorate primary tissue damage and promote repair. Thus, release of such mediators is a first step in creating an environment propitious to regeneration which involves the recruitment of phagocytic and neuronal precursor cells to the site of injury (Streit, 2002; Neumann *et al.*, 2009).

There is accumulating evidence suggesting a role for these mediators in the regeneration of brain tissue after injury. These include cytokines in a soluble form for auto- and paracrine signaling or membrane-associated for cell-to-cell interaction, and which participate in a multitude of biological processes by serving cellular communication. Indeed, several studies show that microglia protect neurons in damaged brain by secreting anti-inflammatory cytokines and growth factors like IL-10, TGF-β and BDNF (Streit, 2005). TGF-β appears to act as an autocrine mediator by regulating negatively inflammatory and immunoregulatory functions of activated microglia, such as suppresion of cytokine production and ROS formation (Suzumura *et al.*, 1993). Also, pro-inflammatory cytokines, such as IL-1β, il-6 and TNF- α, can also be released to play a role in neuroprotection (Imai *et al.*, 2006). In fact, TNF-α released by activated microglia is suggested to induce expression of potent angiogenic factors that promote retinal neovascularization during post-ischemic inflammation (Yoshida *et al.*, 2004). Also, injection of exogenous microglia itself protected CA1 pyramidal neurons against ischemia-induced neuronal degeneration possibly through a neurotrophin-dependent mechanism (Hayashi *et al.*, 2006; Imai *et al.*, 2006).

Neuroprotection by microglia also involves their ability to uptake excitotoxic glutamate by expressing glutamate transporter 1 (Nakajima *et al.*, 2001b). Interestingly activation of this transporter by glutamate leads to production of neurotrophic factors by microglia via PKC pathway (Liang *et al.*, 2010).

In fact, microglia contribute to host defense and repair with immune cytokines acting as neurotrophic substances, protecting and promoting neurite growth during neurodevelopment and under most acute conditions. However, with intense activation, these cytokines and other mediators released by microglia can be very destructive leading to the establishment and maintenance of brain damage (Minghetti and Levi, 1998; Blaylock, 2004). Secretory components include proteinases, ROS and nitrogen intermediates, chemokines, pro-inflammatory cytokines and excitotoxins, such as glutamate (Banati *et al.*, 1993). IL-1β and TNF-α are two pro-inflammatory cytokines, early and prominently produced by microglia upon activation, indicated to have a critical role in neuropathologies. Indeed, TNF-α lies at the beginning of a signal cascade that can lead to neuronal cell death. Also, this cytokine potentiates glutamate neurotoxicity and can stimulate the production of IL-1β, IL-6 and other cytotoxic cytokines (Lee *et al.*, 1993; Chao and Hu, 1994). Thus, cytotoxic properties of microglia can be modulated by cytokines themselves. For example, while IFN-γ prime microglia to become activated producing reactive nitrogen intermediates and TNF-α, following LPS

treatment, (Meda *et al.*, 1995), studies using organotypic hippocampal slice cultures demonstrated that they act neuroprotectively when pre-activated with IL-4 (Butovsky *et al.*, 2006).

NO is the mainly free radical produced by murine microglia upon activation by several stimulants like LPS (Tambuyzer *et al.*, 2009). In co-cultures with neurons, microglia production of ROS lead to the reduction of neurite growth during brain development (Thery *et al.*, 1991). Also, NO induces glutamate release from neurons that leads to NMDA receptors activation and consequent excitotoxicity (Brown, 2010). Years of research using LPS model of microglia activation, have shown that LPS acts on the specific microglial receptors TLR4 and Mac-1 triggering a signaling pathway that results in pro-inflammatory gene expression mediated by NF-κB activation and neuronal death (Loane and Byrnes, 2010).

Microglia are also a source of matrix MMPs, namely MMP-2 and MMP-9 which expression can be induced by LPS stimulation. These enzymes have been shown to degrade components of the basal lamina, leading to the disruption of the BBB and thus, contributing to the neuroinflammatory response in many neurological diseases (Rosenberg, 2002).

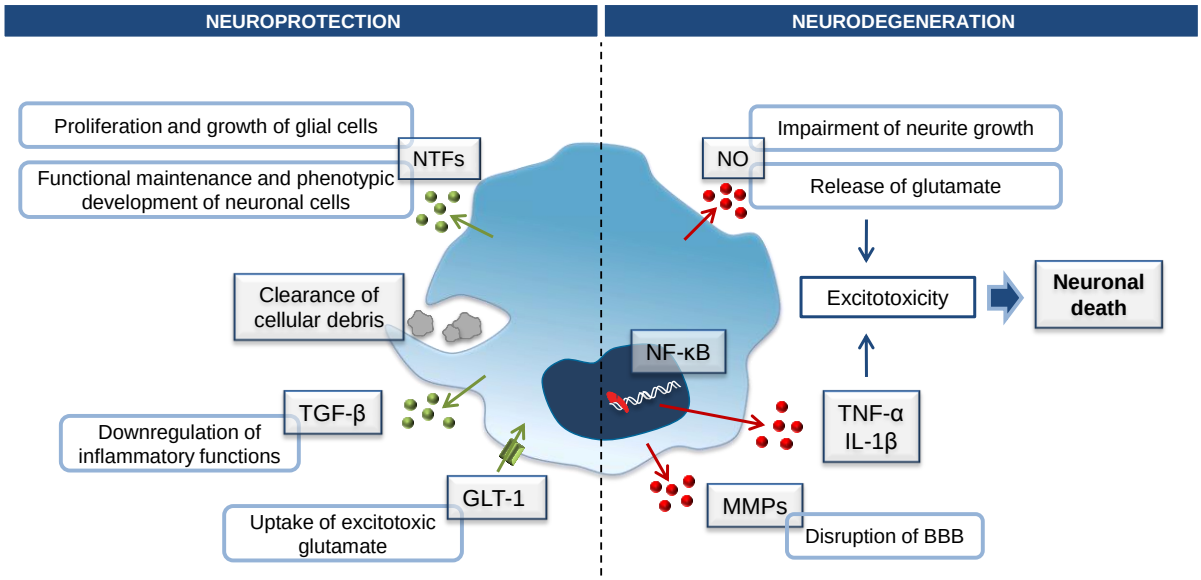


**Fig. I. 8. Examples of mediators produced in neuroprotection and neurodegeneration.**
Microglia exert neuroprotective functions through the production of neurotrophic factors (NTFs) and by the clearance of cell debris, during brain development and under pathological conditions,. Also, they express a glutamate transporter (GLT-1) responsible for the uptake of excitotoxic glutamate. In certain conditions, activation of microglia can exert neurotoxic functions by producing nitric oxide (NO) that intertferes with neurite growth and contributes to the release of glutamate. Also, pro-inflammatory gene expression by nuclear factor (NF)-κB result in pro-inflammatory cytokines production, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Release of these mediators contributes to excitotoxicity resulting in neuronal death. In addition, metalloproteinases (MMPs) are released leading to the disruption of the blood-brain barrier.

Recalling the issue of the different states of activation of microglia and their corresponding functions, we can, in a very simplistic way, define these cells as anti-inflammatory M2 cells or pro-inflammatory M1 cells. However, as already mentioned, microglia response to injury is complex and multifaceted. Nevertheless, it should be always kept in mind that microglia primary purpose is to limit

further damage and restore normal homeostasis, although excessive activation can cause secondary damage leading to aggravation of the pathological scenario.

## 3. Microglia involvement in neurological diseases

Microglia are involved with the onset and progression of the inflammatory response within the brain (Chew *et al.*, 2006) so that unregulated response or over-activation of microglia can have disastrous neurotoxic consequences (Block and Hong, 2005). However, microglia may also exert neuroprotective functions in several pathological conditions.

### 3.1. Acute *vs*. chronic neuroinflammation

Within the context of the CNS, acute reactions occur as a response to limit neuronal damage, as already mentioned, and involve the activation of glial cells, a process also referred as "reactive gliosis", without BBB breakdown or concomitant leukocyte infiltration. Chronic inflammation is generally associated with a CNS disease context rather than CNS injury (Streit *et al.*, 2004a). Since brain is not an immune privileged organ, as originally described, neurodegenerative diseases are characterized not only by local inflammation from resident cell types in the brain, but also by infiltration of peripheral immune cells. However, while infiltrating peripheral immune cells can represent a factor for neuronal toxicity, not always is associated with neurotoxicity, suggesting that local glial cells play a critical role in the inflammatory response (Block and Hong, 2005). Indeed, peripheral adaptive immune system has a key role in the defense of CNS against insults by the production of cytokines that modulate microglial functions (Schwartz *et al.*, 2006). Since infiltration of peripheral immune cells is restricted in the absence of infection or injury, CNS-resident cells such as microglia represent the first line of defense against invading pathogens and potentially provide innate immune signals leading to subsequent adaptive immune responses. These infection-causing microorganisms are recognized by TLRs and trigger the release of pro-inflammatory cytokines, as well as the up-regulation of cell surface molecules involved in the initiation of adaptive immune responses to pathogens (Olson and Miller, 2004; Parkhurst and Gan, 2010).

In acute CNS injury, microglial cells migrate towards the site of lesion where they exert their functional roles. However, whether these functions are pro-regenerative, like phagocytosis and neurotrophic factor production, or neurodegenerative, like the release of pro-inflammatory mediators, differs on the condition. Nevertheless, even though acute insult may trigger oxidative and nitrosative stress, it is typically short-lived and unlikely to be detrimental to long-term neuronal survival (Frank-Cannon *et al.*, 2009).

In contrast, chronic activation is a long-standing and often self-perpetuating neuroinflammatory response that persists long after an initial injury or insult and is, therefore, an important component of the neurodegenerative diseases (Frank-Cannon *et al.*, 2009). Microglia can become chronically activated by either a single, or multiple stimuli, resulting in cumulative neuronal loss (Lull and Block, 2010). Neuronal damage triggers neighboring microglia activation and the production of pro-inflammatory mediators that lead to more cellular death, representing an interminable cycle of progressive neuronal loss (McCarty, 2006). Neurodegenerative CNS disorders, including AD and

Parkinson's disease (PD), are associated with chronic neuroinflammation and elevated levels of several cytokines. Therefore, rather than serving a protective role as does acute neuroinflammation, chronic neuroinflammation is most often detrimental and damaging to the nervous tissue (Frank-Cannon *et al.*, 2009). The mechanisms by which neuronal damage activates the chronic and deleterious microglial inflammatory response appear to involve a combination of several factors released by neurons that produce multiple alterations in the extracellular matrix (Bonneh-Barkay and Wiley, 2009). For instance, recently, it was proposed that the interaction between high-mobility group box 1 (released from inflamed microglia and/or degenerating neurons) with microglial Mac1, sustain chronic inflammation and mediate chronic dopaminergic neurodegeneration, in PD. This, results from the activation of NF-κB pathway and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase which leads to the production of multiple inflammatory and neurotoxic factors (Gao *et al.*, 2011).

Interestingly, several studies demonstrated that microglia can be activated by a pro-inflammatory stimulus in the absence of neuron damage, if the regulatory mechanisms that maintain the homeostasis and prevent microglia from becoming deleterious are deregulated. Therefore, excessive activation could only occur in response to a potent stimulus where these mechanisms are overwhelmed (Block and Hong, 2007). An alternative explanation is that microglia could become "primed" by a previous stimuli so that an additional insult results in an exaggerated and prolonged inflammatory response (Dilger and Johnson, 2008). Indeed, occurrence of nonspecific systemic infection or inflammation is associated with higher risks of prevalence of neurodegenerative diseases suggesting a crosstalk between systemic inflammation and microglia activation in the CNS (Perry *et al.*, 2003; Perry *et al.*, 2010). Also, the aging process may serve as a "priming" stimulus for microglia and a secondary injury will amplify their response explaining the majority of neurodegenerative diseases in later life, such as PD (Block and Hong, 2007; Dilger and Johnson, 2008).

Once again, polarization of microglial cells, which is determined by the environment, will also determine the response to different pathological conditions. M1 macrophages drive the pathogenesis of both types of disease through common mechanisms as well as through disease-specific mechanisms (Saijo and Glass, 2011). For instance, after spinal cord injury, microenvironment favors cytototxic M1 polarization with only a transient appearance of M2 macrophages, early after injury. Whether these cells retain some M2 characteristics, return to a resting, M0 state or remain mostly cytotoxic is still not known (David and Kroner, 2011). An opposite scenario is found during progression of gliomas. Wu and his colleagues (2010) demonstrated that glioma cancer stem cells, present in malignant gliomas, recruit and polarize the macrophages/microglia to become immunosuppressive. Recent information corroborates the view that TAMs are polarized M2 macrophages/microglia and therefore capable of participating in circuits that regulate tumor growth and progression, adaptative immunity, stroma formation and angiogenesis (Mantovani *et al.*, 2002). However, the onset of gliomas has the contribution of M1 macrophages for the creation of a pro-inflammatory environment that is mutagenic and promotes growth (Saijo and Glass, 2011).

In summary, although classical activation of microglia may lead to neurotoxicity, this response is essential for the defense from extracellular pathogenic organisms. Also, alternative activation, as

shown above, may have detrimental outcome. Therefore, an appropriate balance needs to be achieved between M1 and M2 polarization states

From a therapeutic point of view, knowledge about production of specific forms of microglial activation could be the key in turning a pathogenic cell into a therapeutic modality. For instance, it was recently described that PS-containing liposomes can mimic the effects of apoptotic cells on phagocytes to induce the secretion of PGE2, and then shift microglia and macrophages from pro- to anti-inflammatory phenotype by an autocrine action of this growth factor (Wu and Nakanishi, 2011).

### 3.2. Neonatal hyperbilirubinemia

Bilirubin is the final product of heme degradation. At physiologic pH, bilirubin is insoluble in aqueous solution and binding to albumin is required for transportation from blood to the liver. After conjugation in the liver, it is excreted in bile (Kapitulnik, 2004). Presence of high levels of unconjugated bilirubin (UCB), a frequent condition in the neonatal period, may cause encephalopathy. Newborns produce bilirubin at a rate of more than twice the production in adults, because of an increased red blood cell turnover and an immature hepatic clearance (Ostrow *et al.*, 2004). As a consequence, UCB levels may exceed the binding capacity of albumin and the “free”, unbound, bilirubin deposits in the skin and mucus membranes giving to the newborns a typical yellow coloration, called neonatal jaundice (Porter and Dennis, 2002). This temporary “physiologic” jaundice that usually resolves after the first week of life, may have neuroprotective effects if the concentrations of UCB are only slightly elevated, favoring the antioxidant properties of the compound (Kapitulnik, 2004). More elevated concentrations that some infants manifest, may lead to the deposition of this pigment in selected regions of the brain, originating *kernicterus* and even death. Despite the severity of *kernicterus,* the prevalence of this condition is extremely low and what is turning to be important is the possible association between severe jaundice and long-term neurological defects (Brites, 2011). In fact, recent data shows an increased prevalence of mental disorders, like schizophrenia, and an affected cognitive ability in early adulthood (Newman and Maisels, 1990; Maimburg *et al.*, 2008) with the neonatal jaundice condition.

#### 3.2.1. Molecular basis of microglial response to UCB

Several cellular aspects are affected by UCB. These include alterations of cell morphology (Brito *et al.*, 1996; Brito *et al.*, 2000), disruption of plasma membrane integrity and functionality (Brito *et al.*, 2001; Brito *et al.*, 2002; Rodrigues *et al.*, 2002a; Rodrigues *et al.*, 2002b), impairment of endocytosis (Silva *et al.*, 2001a), inhibition of both DNA and protein synthesis (Greenfield and Majumdar, 1974; Yamada *et al.*, 1977), as well as of protein phosphorylation (Hansen *et al.*, 1996). It begins to be clarified the cellular functions that are mainly affected in different CNS cells and thus, in what extent each cell-dependent injury will contribute to the neurodevelopmental outcome of UCB encephalopathy.

Susceptibility to UCB is dependent of both cell type and its differentiation state (Fig. I. 9). These differences are associated with distinct responses to UCB toxicity depending on the pathways involved. In terms of cell type, neurons present a higher rate of cell death both by necrosis and

apoptosis whereas astrocytes reveal impaired glutamate uptake , as well as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) metabolism (Silva *et al.*, 2002). Inhibition of glutamate uptake results in its permanence in the synaptic cleft leading to NMDA-mediated excitotoxicity (Grojean *et al.*, 2000). Neurons are more vulnerable to oxidative stress that astrocytes because of lower glutathione stores (Brito *et al.*, 2008b). This oxidative injury comprises several alterations, like protein oxidation, lipid peroxidation and ROS formation (Brito *et al.*, 2004). Also, there is a deregulation of the energy metabolism with collapse of the inner-mitochodrial membrane potential and ATP release (Vaz *et al.*, 2010). Thus, although astrocytes are still affected by UCB, neurons are more susceptible to mechanisms leading to irreversible damage for which it could account a favored binding of UCB to neuronal cells and also a higher expression of efflux pumps on astrocytes (Silva *et al.*, 2002). Exposure of astrocytes and microglia to UCB initiates an inflammatory response that involves TNF-α and IL-β pathways followed by mitogen-activated protein kinases and NF-κB signaling cascades culminating in the production of TNF-α and IL-β, accumulation of extracellular glutamate and consequent cell death (Fernandes *et al.*, 2006; Gordo *et al.*, 2006). Interestingly, this inflammatory response in microglia is preceded by a phagocytic phenotypes in which microglia attempt to restrain the extension of lesion. Only after prolonged exposure to UCB, microglia start releasing pro-inflammatory mediators (Silva *et al.*, 2010). Interestingly, levels of TNF-α and IL-β released by these cells are much higher than those secreted by astrocytes and even more if compared to neurons (Gordo *et al.*, 2006). Microglia are, therefore, the most reactive cells to UCB and consequently may aggravate neurotoxicity.

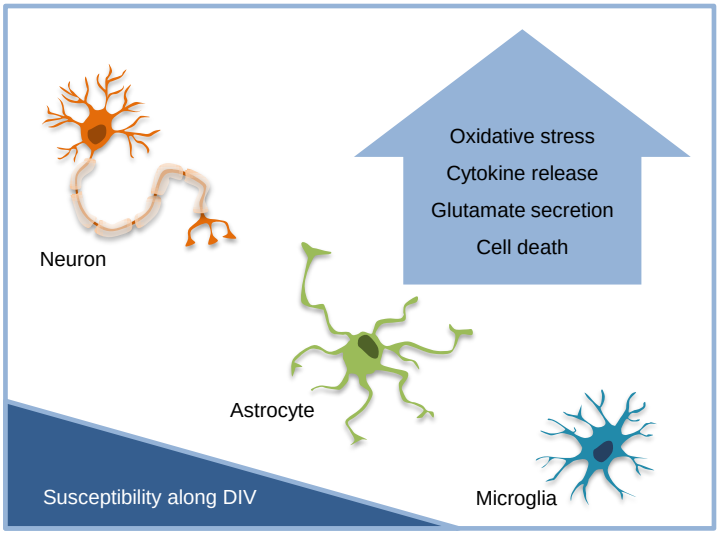


**Fig. I. 9. General molecular mechanisms of unconjugated bilirubin (UCB)-induced neurotoxicity.**
UCB triggers several toxic-related molecular mechanisms in nerve cells. The vulnerability to the deleterious effects of UCB depends on the maturation stage so that it increases with days *in vitro* (DIV).

Regarding the maturation stage of cells, it appears that the more immature cells are the higher vulnerability to UCB-mediated neurotoxicity is observed. Contributing to the susceptibility of immature nerve cells to UCB is the reduced expression of the multidrug resistance-associated protein 1 transporter. This protein limits the accumulation of UCB within the cells by its active export and its

expression increases with cell differentiation (Falcão *et al.*, 2007a). Accordingly, younger astrocytes are more vulnerable to cell death, glutamate efflux and inflammatory response than older ones (Falcão *et al.*, 2005).

Exposure of neural precursors to UCB leads to a decrease in viability and neurogenesis of proliferating cells as well as to an increase in cellular dysfunction of differentiating cells. Thus, these deleterious effects of UCB in the developing CNS may compromise performance of brain in later life. Indeed, exposure of immature neurons to UCB leads to reduction of both neurite extension and number of nodes, effects that are increasingly perpetuated along cell differentiation and exacerbated if exposed later to an inflammatory agent (Falcão *et al.*, 2007b). UCB also interferes with axonal growth, cone area and spine formation (Fernandes *et al.*, 2009), further confirming that its effects in the developing brain may lead to decreased synaptic connectivity, which may underlie the emergence of neurodegenerative diseases. Furthermore, by damaging olygodendrocytes, UCB may compromise myelin biogenesis and repair and, consequently, axonal function. The microenvironment influences cellular response to UCB, particularly of microglial cells. Recent findings revealed that conditioned media derived from UCB-treated astrocytes reduce microglial inflammatory reaction and cell death, probably in an attempt to constrain microglial over-activation. Furthermore, damaged neurons may signal microglial clearance functions by releasing soluble factors, but they can also overstimulate their inflammatory potential, by promoting the release of IL-6 and NO, as well as MMP-9 activation, leading to their demise (Silva *et al.*, 2011). The outcome of nerve cell response to UCB is summarized in Fig. I. 8.

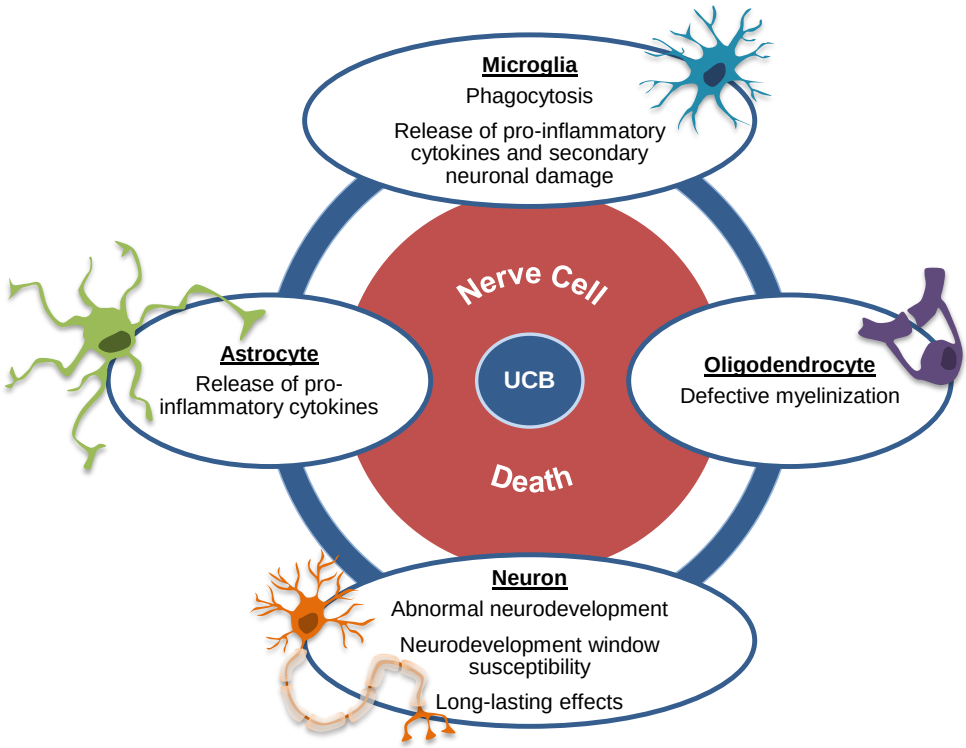


**Fig. I. 10. Contribution of each cell-dependent injury to the general outcome of unconjugated bilirubin (UCB) toxicity.**
Unconjugated bilirubin (UCB) has evidenced toxicity for all the represented cell types. These effects may culminate in nerve cell death, both by apoptosis and necrosis. *Based on Brites, 2011.*

Concerning therapeutic strategies for preventing UCB-induce neurotoxicity, several compounds have already been tested with beneficial results. Since bile salts can stimulate biliary excretion of organic anions, including bilirubin in rats (Cuperus *et al.*, 2009), it was suggested that administration of such compounds could be relevant for the treatment of unconjugated hyperbilirubinemia. Indeed, dally administration of ursodeoxycholic acid, induced a rapid and sustained decrease in plasma UCB concentrations in Gunn rats by stimulation of UCB turnover and its fecal disposal (Cuperus *et al.*, 2009). Furthermore, this hydrophilic bile salt was shown to inhibit mitochondrial membrane instability and release of cytochrome c, preventing apoptosis (Silva *et al.*, 2001b; Rodrigues *et al.*, 2002a). Recent studies demonstrated that the antioxidant compound glycoursodeoxycholic acid (GUDCA) and the immunomodulatory cytokine IL-10 are capable of suppressing UCB pro-inflammatory proprieties (Fernandes *et al.*, 2007; Fernandes and Brites, 2009). Additionally, GUDCA significantly prevented UCB-induced oxidative stress, metabolic alterations, like disruption of glutathione redox status and cell demise in neurons (Brito *et al.*, 2008a; Vaz *et al.*, 2010). Regarding microglial cells, both GUDCA and IL-10 reduced accumulation of extracellular glutamate and counteracted the impairment of neurite outgrowth and ramification, as well as cell death in UCB-treated neurons. However, only GUDCA prevented the UCB synaptotoxicity from occurring (Silva, 2010), pointing out this molecule as a promising therapeutic agent in the management of neonatal hyperbilirubinemia.

## 4. Aims

This work was initially elaborated to evaluate the effect of UCB on eithet basal microglial migration or stimulated microglia, by using ATP or S100B, well-known chemoattractants of these cells. Once GUDCA has already revealed to have antioxidant, immunomodulatory and anti-apoptotic properties, we also aimed to assess its modulation ability on the effects produced by UCB on microglial migration.

When facing the variations between results obtained in different microglia cultures, we hypothesized that we were dealing with a variety of cell phenotypes, which under “stressed” conditions would respond differently to UCB interaction. This led us to also characterize microglial phenotypes in cultures during time *in vitro.*

Therefore, the aims of the present thesis encompass:

**1)** Chemotaxis assays to evaluate microglial migration, in what concerns: **(i)** the potential of “free” unbound UCB as a chemotactic agent; **(ii)** the capacity of migration of UCB-treated microglia; and **(iii)** the modulation capability of GUDCA on UCB-treated microglia;

**2)** Characterization of microglial cultures over time *in vitro* to identify microglial phenotypes.

# II. MATERIALS AND METHODS

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## 1. Materials

### 1.1. Chemicals

Dulbecco's modified Eagle's medium-Ham's F12 medium (DMEM-Ham's F-12), fetal bovine serum (FBS), L-glutamine, sodium pyruvate and nonessential aminoacids were purchased from Biochrom AG (Berlin, Germany); fibronectin, antibiotic-antimycotic solution (Ab/Am) (20X), human serum albumin (HSA; fraction V, fatty acid free), bovine serum albumin, ATP, Hoechst 33258 dye, biotinylated, fluorescent latex beads 1 μm (2.5%) and were from Sigma Chemical Co. (St. Louis, MO). UCB was also obtained from Sigma and purified according to the method of McDonagh and Assisi (1972). Glycoursodeoxycholic acid (GUDCA) (minimum 96% pure) was obtained from Calbiochem (Darmstadt, Germany); (Darmstadt, Germany); Trypsin/Ethylenediamine tetraacetic acid (EDTA) solution (0.25% trypsin, 1 mM EDTA in Hank's balanced salt solution) was purchased from Invitrogen Corporation™ (Carlsbad, CA, USA); DPX mountant for microscopy was obtained from BDH Prolabo (Poole, UK); L-glutamic acid kit, Triton X-100 were obtained from Roche Diagnostics Deutschland GmbH (Mannhein, Germany); S100B was kindly provided by Dr. Cláudio Gomes, from Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa.

All the other chemicals were of analytical grade and were purchased from Sigma or Merck.

### 1.2. Antibodies

Rabbit anti-p65 NF-κB subunit was from Santa Cruz Biotechnology® (Santa Cruz, CA, USA) and goat anti-ionized calcium-binding adaptor molecule 1 (Iba1) was from Abcam (Cambridge, UK); mouse anti-glial fibrillary acidic protein (GFAP) was from Novocastra (Newcastle Tyne, UK) and rabbit anti-microtubule-associated protein 2 (MAP-2) was from Covance (Princeton, NJ, USA); secondary antibody chicken Alexa Fluor 594 anti-goat IgG was purchased from Invitrogen Corporation (Carlsbad, CA) and horse FITC-labeled anti-mouse was from Vector Laboratories (Burlingame, CA, USA); goat anti-rabbit cyanine dye (CY)2, goat anti-mouse CY3 and anti-rabbit horseradish peroxidase conjugated anti-IgG were acquired from GE Healthcare (Chalfont St. Giles, UK).

### 1.3. Equipment

A 48-well microchemotaxis chamber and polycarbonate track-etch membranes with polyvinylpyrrolidone (PVP) treatment were purchased from Neuro Probe, Inc (Gaithersburg, MD, USA). Leica DFC490 camera adapted to an AxioSkope® microscope were from Carl Zeiss, Inc., (North America).

## 2. Methods

### 2.1. Primary culture of microglia

Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used and their suffering.

Mixed glial cultures were prepared from 1-to-2 day-old CD1 mice as previously described (McCarthy and de Vellis, 1980), with minor modifications (Gordo *et al.*, 2006). Cells ($4 \times 10^5$ cells/$cm^2$) were plated on uncoated 12-well tissue culture plates (with 18 mm coverslips) or 75-$cm^2$ culture flasks in culture medium (DMEM-Ham's F-12 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids 1X, 10% FBS, and 1% antibiotic-antimycotic solution) and maintained at 37ºC in a humidified atmosphere of 5% $CO_2$.

Microglia were isolated as previously described by Saura et al. (2003). Briefly, after 21 days in culture, microglia were obtained by mild trypsinization with a trypsin-EDTA solution diluted 1:3 in DMEM-Ham's F12 for 45 to 60 min. The trypsinization resulted in detachment of an upper layer of cells containing all the astrocytes, whereas the microglia remained attached to the bottom of the well. The medium containing detached cells was removed and replaced with initial mixed glial-conditioned medium. The use of 21 days *in vitro (*DIV) cells intents to achieve the maximal yield and purity of the cultures. In fact, astrocyte contamination was less than 2%, as assessed by immunocytochemical staining with a primary antibody against GFAP followed by a species-specific fluorescent-labeled secondary antibody and also there was no neuron contamination, as assessed by immunocytochemical staining with a primary antibody against MAP-2 followed by a species-specific fluorescent-labeled secondary antibody (Silva *et al.*, 2010).

### 2.2. Migration Assay

Twenty-four hours after microglia trypsinization, attached cells on uncoated 75-$cm^2$ flasks were subjected to different treatments. After these incubations, isolated microglia were recovered by a 10-min incubation with pure trypsin-EDTA solution with subtle scratching and vigorous pipetting. Chemotaxis assays were performed using this cell suspension (Fig. II.1).

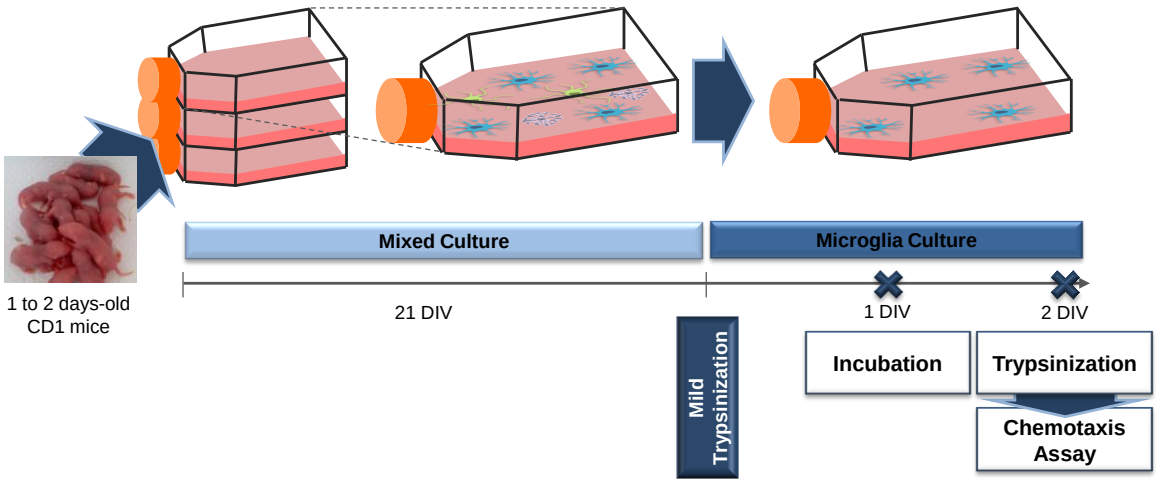


**Fig. II. 1. Schematic representation of the experimental model for chemotaxis assays.**
Mixed glial cultures were prepared from 1-to-2 day-old CD1 mice and plated on 75-cm$^2$ culture flasks in culture medium. After 21 days *in vitro* (DIV), isolated microglia was obtained by mild trypsinization. At 1 DIV, cells were subjected to the different treatments. After 24 h-incubations, isolated microglia were recovered by tripsinization followed by subtle scratching and vigorous pipetting. Chemotaxis assays were performed in using this cell suspension.

### 2.2.1.Cell treatments

Microglial cells were incubated with DMEM-Ham's F12 without (control) or with 50 µM UCB, in the presence of 100 µM HSA for 24 h, at 37ºC. In parallel experiments, cells were pre-incubated with 50 µM GUDCA (from a 5 mM stock solution), 1 h prior to UCB addition.

To study the role of free, unbound UCB ($_f$UCB) as a chemoattractor, microglia were incubated with DMEM-Ham's F12 only.

The UCB stock solution (10 mM) was prepared in 0.1 M NaOH immediately before use and the pH of the incubation medium was restored to 7.4 by addition of equal amounts of 0.1 M HCl. All the experiments with UCB were performed under light protection to avoid photodegradation.

### 2.2.2.Establishment of the microchemotaxis assay

Implementation of the microchemotaxis assay was an especially difficult and time-consuming process. We have originally adopted the protocol reported by Färber *et al.* (2008) but, after several attempts and failures, several modifications were made in order to achieve optimal results (Fig. II. 2).

Cell migration assays were performed in a 48-well microchemotaxis chamber. The bottom wells of the Boyden chamber were loaded with solutions described in Table II.1. A PVP-treated polycarbonate filter (8 µm pore size) was washed with 0.02 N acetic acid to remove PVP treatment and coated with 10 mg/ml fibronectin in PBS overnight as described by Honda *et al.* (2001) and installed in the microchemotaxis chamber.

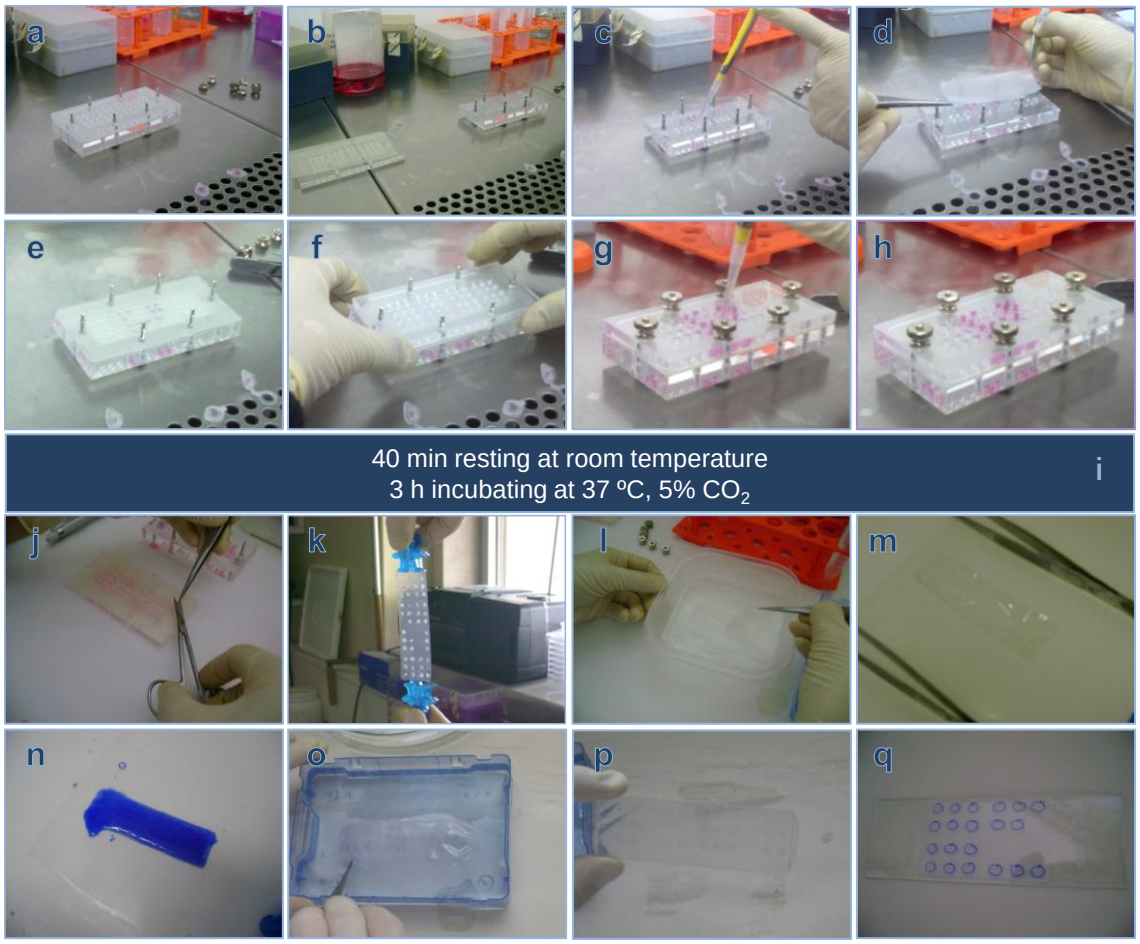


**Fig. II. 2. Representative steps of the chemotaxis assay.**
**a-b.** Microchemotaxis chamber is disassembled (thumbnuts, top plate and gasket are removed); **c.** solutions of potential chemoattractants are added to the lower wells (in the bottom plate); **d.** A coated filter is placed on top of bottom chamber; **e-f.** Chamber is assembled (gasket and top plate are placed over the filter and thumbnuts are tightened); **g.** Cellular suspension is applied into the upper wells (in the top plate) **h.** Microchemotaxis chamber is prepared; **i.** Chamber is left for 40 min at room temperature and then incubated for 3h at 37ºC and 5% $CO_2$; **j.** Chamber is disassembled and, with cells facing up on the filter, a cut is made on the top right corner of the filter for orientation; **k.** Non-migrated cells are removed with a cotton swab soaked in PBS; **l.** Cells are fixated with methanol; **m.** Filter is placed with cell-side up on a disposable lint-free towel for air-drying; **n.** For staining, Giemsa solution is infiltrated under the filter with cell-side down; **o.** Filter is rinsed with distilled water; p. Filter is placed on a glass slide for air-drying; q. Filter is mounted with Entellan between slide and coverglass and wells are marked on the slide.

**Table II. 1. Solutions loaded to the bottom wells of the microchemotaxis chamber.**

| Condition | Solution |
|---|---|
| **Vehicle** | DMEM-Ham's F-12 |
| **Described chemoattractants** | ATP (300, 100 and 10 µM)<br>S100B (1 µM) |
| **Potential chemoattractants** | $_f$UCB (70 and 140 nM) |

After the incubation procedures, described in 2.2.1., microglia were suspended in serum-free DMEM-Ham's F-12 and 50 µL of cell suspension was placed into each top well (3 x $10^4$ cells/well).

The Boyden chamber was left at room temperature for 40 min, in order for cells to settle and to improve even distribution on the filter as described by Miller and Stella (2009), and then placed in a $CO_2$ incubator at 37°C for 3 hours. The filter was removed and cells on the top side of the filter were wiped off with cotton-tipped swabs soaked in PBS. Filter was then fixed with methanol and stained with freshly prepared and filtered 10% Giemsa in PBS according to Chen (2004). Image of one microscopic field (original magnification: 100X) were acquired per well using a Leica DFC490 camera adapted to an AxioSkope® microscope and the number of cells was counted. On each experiment at least three wells were sampled per condition.

### 2.3. Microglia phenotypic characterization

After mild trypsinization, attached cells on uncoated 18-mm coverslips were maintained at 37ºC in a humidified atmosphere of 5% $CO_2$ until reaching 2, 10, 13 or 18 DIV for culture characterization. Culture medium was replaced every 4 days (Fig. II. 3).

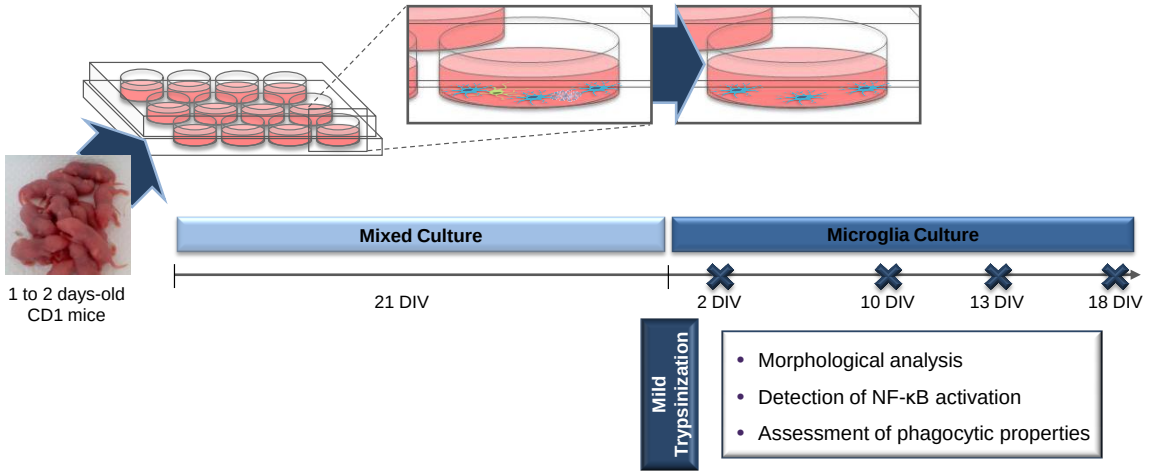


**Fig. II. 3. Schematic representation of the experimental model for culture characterization.**
Mixed glial cultures were prepared from 1-to-2 day-old CD1 and plated on uncoated 12-well tissue culture plates (with 18 mm coverslips) in culture medium. After 21 days *in vitro* (DIV), isolated microglia was obtained by mild trypsinization and cultures were maintained for 18 DIV. At 2, 10, 13 and 18 DIV, we evaluated the parameters indicated above, for culture characterization. NF-κB, nuclear factor-κB.

#### 2.3.1. Morphological Analysis

For morphological analysis, cells with different DIV were fixed for 20 min with freshly prepared 4% (w/v) paraformaldehyde in PBS and a standard immunocytochemical technique was performed using a primary antibody raised against Iba-1 (goat, 1:500) and a secondary Alexa Fluor 594 chicken anti-goat antibody (1:200). To identify the total number of cells, microglial nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized using a Leica DFC490 camera adapted to an AxioSkope® microscope. Pairs of U.V. and green-fluorescence images of ten random microscopic fields (original magnification: 400X) were acquired per sample. The number of round and ramified microglia was counted to determine the percentage of both morphological types at each DIV.

#### 2.3.2. Detection of NF-κB activation

For immunofluorescence detection of NF-κB nuclear translocation, cells were fixed as described above and a standard indirect immunocytochemical technique was carried out using a polyclonal rabbit anti-p65 NF-κB subunit antibody (1:200) as the primary antibody and a anti-rabbit Cy2 as the secondary antibody (1:1000). Microglial nuclei were stained with Hoechst 33258 dye Fluorescence was visualized using a Leica DFC490 camera adapted to an AxioSkope® microscope. Pairs of U.V. and green-fluorescence images of ten random microscopic fields (original magnification: 400X) were acquired per sample. NF-κB positive nuclei (identified by localization of the NF-κB p65 subunit staining exclusively at the nucleus) and total cells were counted to determine the percentage of NF-κB-positive nuclei at each DIV.

#### 2.3.3. Assessment of microglial phagocytic properties

To evaluate the phagocytic capacity of the primary microglial cultures, cells with different DIV were incubated with 0.0025% (w/w) 1 µm fluorescent latex beads for 75 min at 37ºC and fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS. Microglial nuclei were counterstained with Hoechst 33258 dye. Fluorescence was visualized using a Leica DFC490 camera adapted to an AxioSkope® microscope. U.V. and green fluorescence images of ten random microscopic fields (original magnification: 400X) were acquired per sample. The number of ingested beads per cell and total cells was counted to determine the percentage of phagocytic cells and the mean number of ingested beads per cell.

### 3. Statistical Analysis

Results of, at least, three different experiments were expressed as mean ±S.E.M. Significant differences between two groups were determined by the two-tailed t-test performed on the basis of equal and unequal variance as appropriate. Comparison of more than two groups was done by ANOVA followed by Dunnett's multiple comparison test or Tukeys multiple comparison test as appropriate. Statistical analysis weres performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) Statistical significance was considered for a p value less than 0.05.

# III. RESULTS

## III. RESULTS

Microglia are known to play crucial roles in the development and course of neurodegeneration. Due to the immunostimulatory effects of UCB and microglia activation (Fernandes *et al.*, 2004; Gordo *et al.*, 2006) it is expected that microglia will be chemoattracted by soluble factors released by astrocytes or neurons upon exposure to UCB, or even to UCB itself. Extracellular ATP is the main stimulus for activation and early motility of microglia following several injuries (Kurpius *et al.*, 2007; Samuels *et al.*, 2010). In fact, as previously introduced, microglia can transform into mobile phagocytes and rapidly accumulate at sites of neuronal injury, promoting clearance of dead and dying cells and thereby helping to limit secondary injury during hyperbilirubinemia. Recent findings evidenced a switch of this chemotactic response to ATP in activated, or proinflammatory, microglia. Specifically, in LPS-activated microglia, ATP induced process retraction and repulsive migration, effects that are opposite to those seen in unstimulated cells (Gyoneva *et al.*, 2009). Moreover, S100B protein, extremely elevated in the aging brain and in several pathological conditions, also seems to stimulate microglia migration (Bianchi *et al.*, 2011). Thus, in this section it will be evaluated the effects of UCB on microglia migration in the absence or presence of these chemoattractants. GUDCA acid will be the compound assessed as a promising therapeutics to modulate microglia migration, once our lab has demonstrated that GUDCA as immunomodulatory properties, among others (Fernandes *et al.*, 2007; Fernandes and Brites, 2009)

### 1. Assessment of microglial chemotaxis to "free" UCB , evaluation of UCB-treated cells ability to migrate, and evaluation of GUDCA as modulator of microglial migration

This task aimed to evaluate the role of UCB in microglia migration during neonatal hyperbilirubinemia. Microglia chemotaxis is usually evaluated by the scratch assay (Yuskaitis and Jope, 2009) or by transwell /Boyden Chamber assays (Takayama and Ueda, 2005; McHugh *et al.*, 2010). We decided to use only the Boyden Chamber, once the scratch assay did not previously proven to be reproducible in our model.

#### 1.1. Evaluation of the chemoattractive potential of UCB

To explore whether microglial cells showed an increased ability to migrate when stimulated by UCB we selected two different concentrations of "free" UCB (70 nM and 140 nM) that previously revealed to produce neurotoxic effects *in vitro* (Brito *et al.*, 2008b). Different concentrations of ATP (10 and 300 μM) were used to evaluate the most effective as chemoattractant and to allow the interpretation of UCB effects.

As shown in Fig. III. 1, 300 μM ATP strongly stimulated migration as compared to the lower concentration of 10 μM, which did not cause any effect in the number of migrated cells comparatively to the vehicle. Moreover, both concentrations of $_{f}$UCB showed no effect on microglia migration ability. These results demonstrate that, in these conditions, in our model, $_{f}$UCB does not act as a chemotactic agent for these cells indicating an additional prejudicial effect to the UCB-induced neurotoxicity. In fact, instead of promoting the clearance of dead neurons, by "calling" microglia to the site of injury,

$_f$UCB revealed total inability to attract these cells. To notice that the assays revealed a great variability between cultures, probably due to phenotypical diversity of microglia cultures, and therefore, this topic deserves further investigation. Nevertheless, we thought that could be interesting to evaluate if microglia migratory behavior would be altered by exposure to UCB, namely when in the presence of factors usually release by damaged cells, such as ATP and S100B.

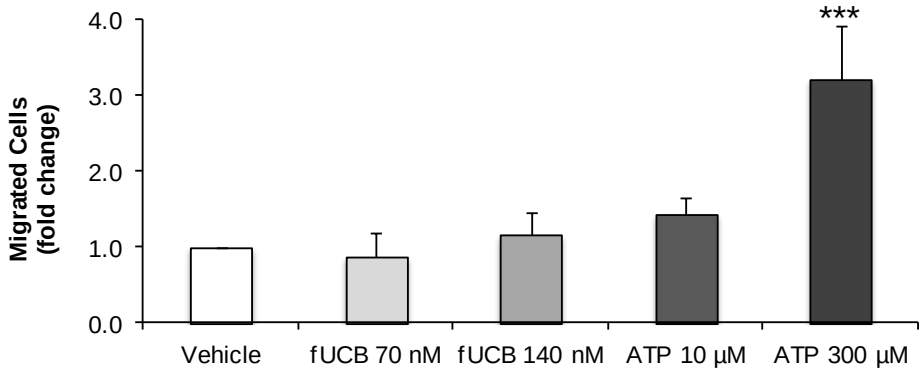


**Fig. III. 1. Free, unbound, unconjugated bilirubin ($_f$UCB) do not induce chemotaxis of microglial cells in the Boyden chamber assay.**
Microglia migration ability was assessed in the absence (vehicle) and in the presence of $_f$UCB (70 and 140 nM) or ATP (10 and 300 µM). Data are shown as the mean ± SEM of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. ***$p$<0.001 *vs.* vehicle

### 1.2. Evaluation of microglia migration ability when treated with UCB

Although migration toward pathological area is the first critical step in microglia engagement during the central CNS injury, we believe that during certain pathological scenarios, these cells might be affected in such a way that they do not accumulate at the lesion site. Although immunosuppressive effect of UCB on chemotaxis of peripheral immune cells has already been described (Vetvicka *et al.*, 1991), the influence of UCB stimulation in this property was never investigated in the brain immune cells, microglia. Thus, we evaluated the migratory ability of UCB-treated cells after a 24 h incubation period.

Exposure of microglial cells to UCB led to a great loss of their migration ability, as represented in Fig. III. 2. We have observed a reduction of approximately 40% in the number of migrated cells comparatively to cells that were not exposed to the compound (control). Again, we wonder whether we had been able to reproduce microglia phenotypes from culture to culture as suggested by the variability of the results, and so, we will surely address this point again in the future.

To assess whether the effect was maintained in the presence of chemotatic agents, we used 300 µM ATP that we previously demonstrated to induce such attraction, as well as 1 µM of S100B, recently indicated to stimulate microglia migration. Interestingly, microglia previously incubated with UCB revealed decay in its migration ability to such compounds (Fig. III. 3) pointing to UCB damage to microglial cells. As in the previous section, UCB diminished microglia chemotaxis ability in more than 50%. Again, this reduced response in UCB-treated microglia, even in the presence of ATP and

S100B, is very interesting in revealing a new pathophysiological mechanism that UCB may have in neurological injury produced during *kernicterus*. Therefore, to confirm this immunosupressive effects of UCB on microglia migration, microglia phenotype should be next characterized and reproduced between experiments. In fact, we were surprised to observe a higher number of control cells migrating to the vehicle than when stimulated with ATP, suggesting a repulsive effect of this compound (data not shown), an effect already described for LPS-activated microglial cells.

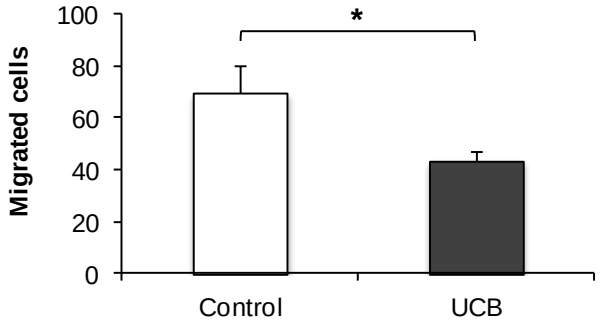


**Fig. III. 2. Unconjugated bilirubin (UCB) exerts immunosuppressive effect on microglia migration.**
The migration assay was performed with non-treated (control) or UCB-treated (50 µM) microglia, as described in Materials and Methods. Data are shown as the mean ± SEM of at least three independent experiments. Statistical analysis was performed by a two-tailed *t* test. **p*<0.05 *vs.*control.

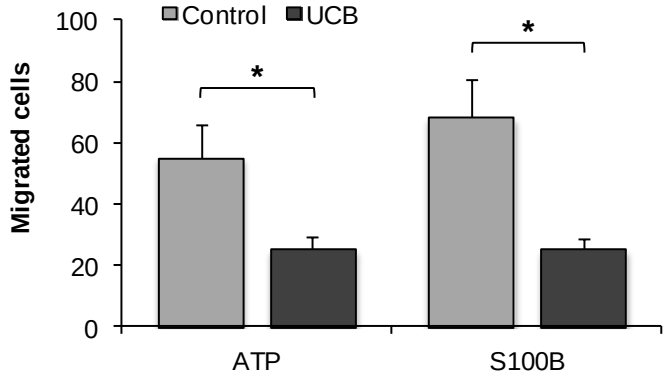


**Fig. III. 3. Immunosuppressive effects of unconjugated bilirubin (UCB) are maintained even in the presence of the chemotactic compounds ATP and S100B.**
The migration assay was performed with non-treated (control) or UCB-treated microglia in the presence of ATP (300 µM) or S100B (1 µM), as described in Materials and Methods. Data are shown as the mean ± SEM of at least three independent experiments. Statistical analysis was performed by a two-tailed *t* test. **p*<0.05 *vs.*control.

### 1.3. Evaluation of GUDCA efficacy in modulating migration of UCB-treated microglia

We observed intriguingly effects of GUDCA on non-treated or UCB-treated microglial cells. Both in the presence of ATP or DMEM-Ham's F12 alone, this bile acid appears to have almost the same effect as UCB (Fig. III. 4). In contrast, when microglial cells were treated with a combination of UCB and GUDCA the results revealed a marked increased stimulation in microglia migration ability, with the number of migrated cells largely surpassing those obtained in all other conditions. This novel modulation by GUDCA may have very important implications in the early stages of UCB-induced

neurotoxicity. This consideration comes from the observation that migratory response decreased in the presence of ATP and S100B, i.e. when the neural cells release such compounds in response to UCB-cytotoxicity. As before, we will have to make proof of concept of such data in the future.

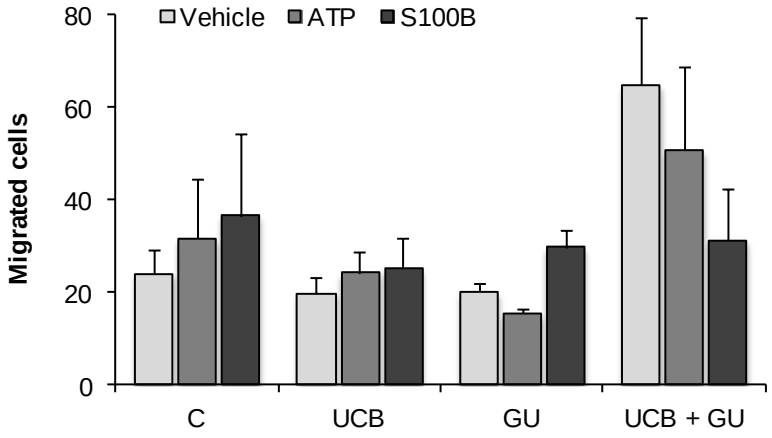


**Fig. III. 4. Glycoursodeoxycholic acid (GUDCA) stimulates cell migration of unconjugated bilirubin (UCB)-treated microglia which is maintained in the presence of ATP and abolished in the presence of S100B.**
The migration assay was performed in the absence and in the presence of ATP (300 µM) or S100B (1 µM), as described in Materials and Methods. Cells were not treated (control) and treated with 50 µM UCB or 50 µM GUDCA, alone or in association Results are shown as fold change ± SEM compared with UCB or GUDCA normalized to control values of three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukeys multiple comparison test.

### 1.4. Critical analysis of the results

We realized that the great variability between experiments as well as the reduced number of migrated cells in all assays may have made impossible to achieve statistical significance in some cases where the results clearly pointed to differences, and therefore, may have compromised the results achieved. We thought that this could be a consequence of the existence of multiple microglia phenotypes, whose representation might be distinct from experiment to experiment. Also, cells could be previously activated as a result of the "stress" caused by the isolation procedure. This idea of an reactive microglia is reinforced by the inability of ATP to stimulate migration of these cells is several experiments. Indeed, it was demonstrated that this compound can induce process retraction and repulsive migration in LPS-activated microglial cells (Gyoneva *et al.*, 2009). Also, even in the activated state, microglial cells from the same culture can have different phenotypes and respond differently to a same stimulus. Furthermore, if microglial cells were indeed activated by the isolation procedure, a subsequent stimulus, like UCB, might have led to their over-activation. Based on that, we decided not to pursue further the experiments without investigating which microglia phenotype we were dealing with.

## 2. Microglial activation profile: Influence of the days in culture

Microglia cultures are a common system employed to explore aspects of microglia phenotype and function. The majority of experiments to study microglia biology are conducted in isolated microglia recently obtained, assuming that they present the same phenotype as the one we would encounter in

their physiological microenvironment. However, a recent study with microglia cultures from rat ventral midbrain demonstrated that the experimental procedures to obtain primary cultures change initial activation status of microglial cells so that they remain in the activated state for several days after the isolation process (Cristóvão *et al.*, 2010). Therefore, when evaluating microglia reactivity to potential toxic compounds one must consider that this reactivity may change depending on the microglia phenotype.

In this section, we established primary cultures of microglial cells from whole brain of newborn mice and we have evaluated the changes in the activation profile of these cells over time in culture.

### 2.1. Microglia morphology changes with the days in culture

Modification of microglial morphology is one of the hallmarks of its activation profile and has been widely used to categorize different activation states (Kim and de Vellis, 2005; Chew *et al.*, 2006). For this reason, we used immunocytochemistry to assess the morphology of microglia from cultures kept for 2, 10, 13, and 18 DIV. More than 98% of the cells in culture were immunopositive for the microglia marker Iba1 (Fig. III. 5A). Amoeboid and ramified cells were counted and our results revealed a gradual increase in the number of ramified cells along time *in vitro* till the 13 DIV (Fig. III. 5B), followed by a slight decrease at 18 DIV, indicating that microglia acquires a more "resting" phenotype at 13 DIV in culture. In fact, ramified cells increased from 2 to 13 DIV, where they achieved the value of 83.2 ± 5.1%, thus with a majority of cells showing a morphology indicative of surveillant microglia. If we look to the number of cells with amoeboid morphology, more characteristic of reactive microglia, we observed a decrease from 31.6 ± 0.6% at 2 DIV to 30.0 ± 5.2% at 10 DIV, achieving the minimum representation of 15.9 ± 5.1% at 13 DIV. Notice that the cultures at 18 DIV showed, again, a novel increase in amoeboid cells (22.7 ± 9%) (Fig. III. 5B). Branched cells from these cultures were typically unipolar or bipolar. The expression and localization of Iba1 in microglial cells has also changed during time *in vitro*. Initially Iba1 besides being localized in the cytoplasm and nucleus, could also be observed in lamellipodia and membrane ruffles. After 18 DIV, expression became weak and localized throughout the microglial processes, with exceptional cases in which Iba1 expression was preferentially perinuclear (Fig. III. 5A). Moreover, nucleus became more elongated and some rod-shaped nucleus morphology could be seen (Fig. III. 5Aa-d) together with a lower cell density, therefore suggesting loss of cell functionality.

### 2.2. Activation state of microglial changes with the days in culture

Microglial cells play key immune-related duties, intervening by producing anti-inflammatory compounds and trophic factors, by phagocytizing non-functional tissue, as well as by releasing cytokines. Production of several cytokines during microglial activation process is associated with the activation of the inducible transcription factor NF-κB. To evaluate the functional behavior of primary microglial cultures, we investigated NF-κB transactivation at the same time points used for analyzing morphological changes. We evaluated the localization, either cytoplasmic or nuclear, of p65 NF-κB subunit by immunocytochemistry (Fig. III. 6A). NF-κB positive and negative nuclei were counted and we observed maximum levels of NF-κB activation at 2 DIV (14.6 ± 3.3%), thus confirming the previous

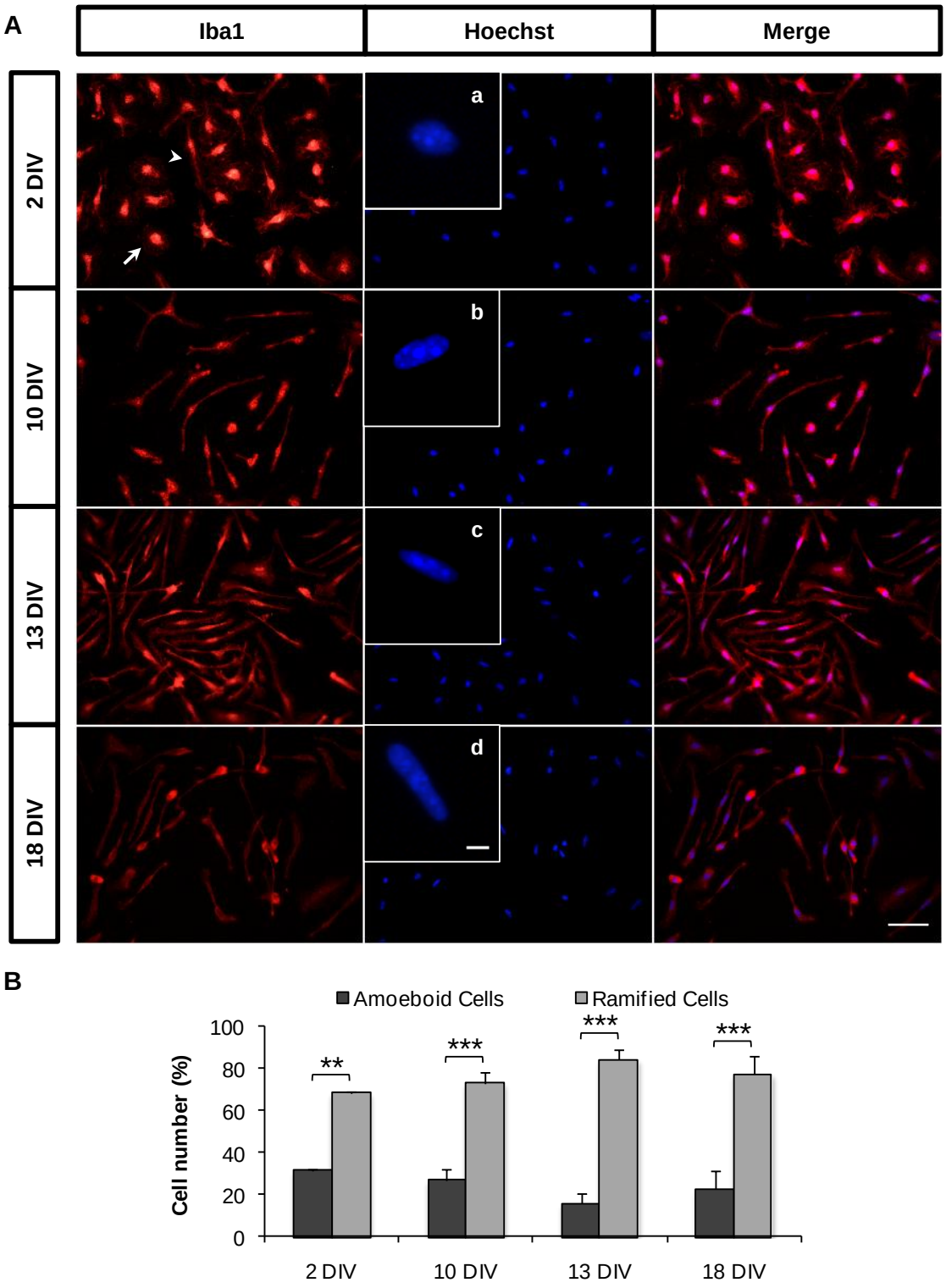


**Fig. III. 5. Microglia morphology evaluation point to 13 days *in vitro* (DIV) cultures as the most suitable for studies of reactivity**

**A.** Microglial cell cultures from mouse cerebral cortex were labeled with Iba1 at different (DIV). Morphological changes occur both in cytoplasm and nucleus of microglial cells (**a – d**). **B.** Quantification of amoeboid (arrow in A) and ramified microglial cells (arrowhead in A) at different DIV. The minimum value of amoeboid morphology was observed after 13 DIV, with the majority of cells showing the ramified morphology characteristic of non-reactive microglia. Data are mean ± SEM from at least four independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test. ***p*<0,01 and ****p*<0.001, comparing the number of amoeboid cells to the number of ramified cells. Scale bar = 50 µm; insets scale bar = 5 µm.

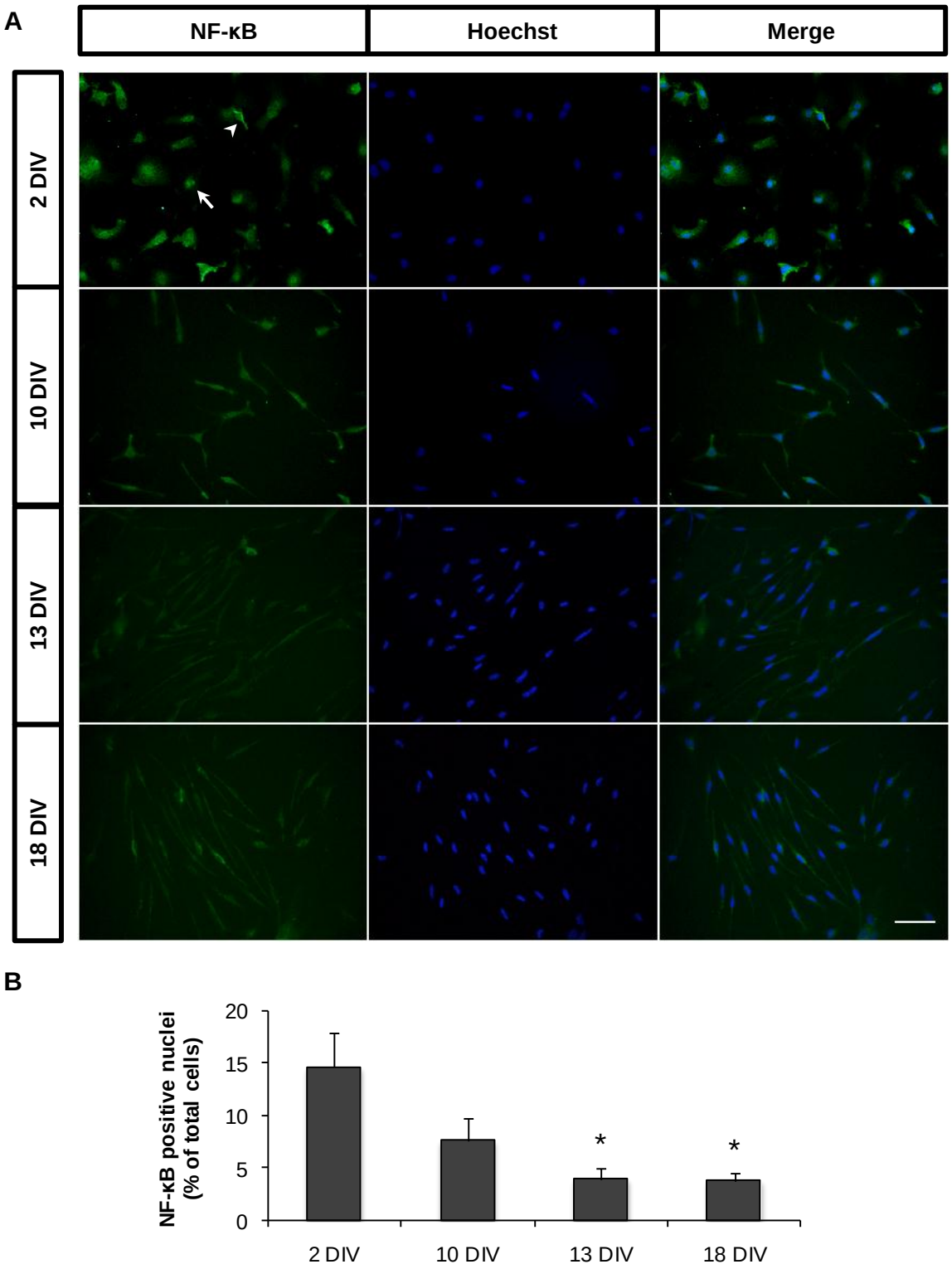


**Fig. III. 6. Microglial cells at 13 *in vitro* (DIV) show the lowest levels of nuclear factor-kappaB (NF-κB) activation.**

**A.** Microglial cells at different DIV were immunostained with an antibody against p65 NF-κB subunit. **B.** Quantification of NF-kB positive (arrow in A) and negative nuclei (arrowhead in A) of microglial cells at different DIV indicate that the less activation is achieved after 13 DIV. Data are mean ± SEM from at least four independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test. *p<0.05 *vs*. 2 DIV. Scale bar = 50 µm.

results showed maximum levels of NF-κB activation at 2 DIV (14.6 ± 3.3%), thus confirming the previous results obtained for morphology and reinforcing that cells are still highly reactive after 2 DIV. NF-κB translocation into the nucleus achieved the minimum levels at 13 and 18 DIV with less than 5% of the cells evidencing NF-κB activation (Fig. III. 6B).

To further characterize the state of activation of these cultures over time *in vitro*, we then evaluated the phagocytic capability of primary microglial cells at early and late time points after plating (Fig. III. 7A). Phagocytosis achieved the lowest values at 10 DIV whereas cultures with 13 DIV showed the best responsiveness to the latex beads (Fig. III. 7B).

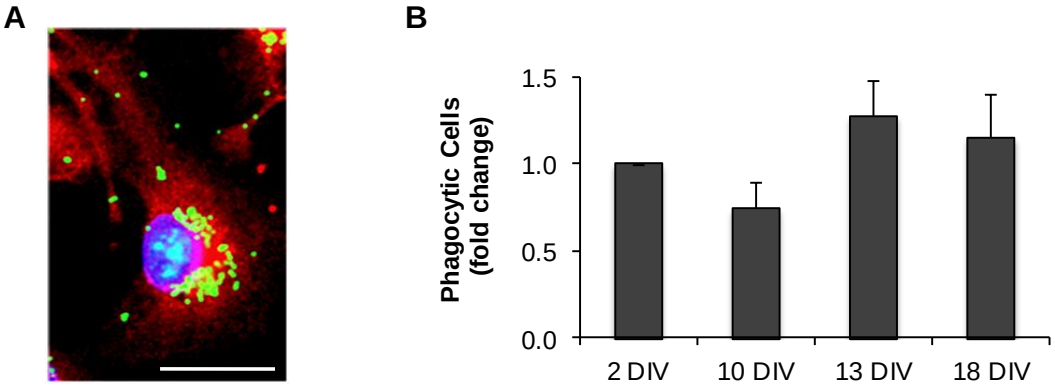


**Fig. III. 7: Microglia phagocytic phenotype achieved maximum capability at 13 days *in vitro* (DIV).**
**A.** Microglial cells at different DIV were incubated with 1 µm fluorescent latex beads as described in Materials and methods. **B.** Quantification of bright, punctuated fluorescent cells at different DIV. Data are shown as the mean ± SEM of at least four independent experiments performed in triplicated. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test. Scale bar = 20 µm.

Interestingly, and in agreement to what we have been pointing lately, we observed a wide diversity in both in terms of number of phagocytic cells (data not shown) and in terms of the number of beads ingested per cell at each DIV. Taking this into account, cultures with 13 DIV show the most equitable representation of the distinct phagocytic behaviors (Fig. III. 8).

**Table III. 1. Microglial cultures with 13 days *in vitro* (DIV) present the best representation of the phagocytic phenotypes variability.**

| Beads | Percentage of cells | | | |
|---|---|---|---|---|
| | 2 DIV | 10 DIV | 13 DIV | 18 DIV |
| 0 | 63.1 | 73.4 | 63.0 | 63.9 |
| 1 | 12.7 | 15.6 | 17.8 | 17.5 |
| 2 | 5.7 | 5.9 | 7.8 | 6.6 |
| 3 | 3.6 | 2.7 | 3.8 | 3.6 |
| 4 | 2.8 | 0.7 | 2.1 | 3.2 |
| 5 | 1.9 | 1.0 | 1.9 | 1.2 |
| 6 | 1.3 | 0.3 | 1.4 | 1.0 |
| 7 | 1.9 | 0.2 | 0.7 | 0.9 |
| 8 | 0.6 | 0.1 | 0.3 | 0.2 |
| 9 | 0.5 | 0.0 | 0.2 | 0.2 |
| ≥10 | 6.0 | 0.1 | 1.2 | 1.6 |

Quantification of the number of phagocytosed beads per cell, at the different DIV.

# IV. DISCUSSION

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In this work we showed that exposure to UCB leads to a reduction of microglia ability to migrate, even in the presence of chemoattractants such as ATP and S100B. Even in the absence of albumin, $_{f}$UCB has no direct effect on chemotactic response of microglia. Interestingly, when incubated with a combination of UCB plus GUDCA, in contrast with either one alone, microglia develops a significant increase in its migration ability. Microglia have shown different phenotypes according with the time of culture, and the 13 DIV revealed to be the best time to obtain the microglia morphology of the "resting" state, together with the less NF-κB translocation to the nucleus and the most equilibrated representation of phagocytic phenotypes, indicating that microglia response to insult should be evaluated at 13 DIV in our model.

The first response of microglial cells to an insult is their rapid accumulation at the site of lesion. This is a consequence of migration and proliferation of these cells regulated by numerous factors released at the local. Indeed, it is hypothesized that a halo of ATP in micromolar range exists even in the steady state and levels of 80–100 μM may be released by astrocytes (Lambert *et al.*, 2010). This purine has been extensively reported as a chemoattractant of microglia as it induces membrane ruffling and chemokinesis of these cells in Boyden chamber assays (Honda *et al.*, 2001), and also leads to activation and migration of microglia *in vivo* (Samuels *et al.*, 2010). Here, we provide another evidence for ATP role as a chemoattractive compound and confirm 300 μM as an effective concentration for stimulation of microglia migration, as already described by Miller and Stella (2009). Furthermore, with this study we started to unveil the role of UCB in the migratory ability of microglial cells, never performed previously. The results we obtained, evidence that $_{f}$UCB has no chemoattractant effect on these cells. Therefore, data suggest that, during neonatal hyperbilirubinemia, distant microglia are not recruited to the site of neuronal injury by $_{f}$UCB, what may additionally damage neurons and glial cells. This is not without precedent since it was demonstrated that UCB inhibits chemotaxis of human polymorphonuclear leukocytes upon stimulation with chemotactic agents (Miler *et al.*, 1981; Vetvicka *et al.*, 1991). Moreover, a recent study showed that one day after intracerebral hemorrhage, there was an increased neutrophil infiltration associated with UCB whereas an opposite effect was seen for microglia that was present in a reduced number at the site of lesion (Loftspring *et al.*, 2011).

Microglia can either decrease or enhance its migratory ability after exposure to stimuli. Therefore, we evaluated how microglia behaves after treatment to UCB when exposed to vehicle and to ATP or S100B. In these conditions it seems that UCB is responsible for immunosuppressive effects on microglia chemotaxis. Only a few papers have previously addressed this issue for other compounds. Our results point to an UCB-mediated reduction in microglia ability to migrate. Moreover, this immunosuppressive effect was maintained in the presence of ATP or S100B. Previous results have shown that 2-chloro-N(6)-(3-iodobenzyl)-5'-N-methylcarbamoyl-4'-thioadenosine (LJ529), a selective $A_{3A}R$ agonist, prevented the infiltration/migration of microglia and monocytes after middle cerebral artery occlusion and reperfusion, or after LPS injection into the corpus callosum. Moreover, using a mouse BV-2 microglial-like cell model, Fraga and his co-workers (2011) demonstrated that the

exogenous cannabinoids Delta-9-tetrahydroca nnabinol (THC) and CP55940 exerted a concentration-related reduction in the migration of BV-2 cells. Effects of UCB at the level of membranes (Brito *et al.*, 2004) may trigger the redistribution of receptors, thus changing microglia motility response.

A direct interaction of UCB molecules or UCB-albumin complexes may be a primary cause of the observed effects. The binding to cell membranes might significantly alter the cell membrane properties and thus change the normal functions of the membrane structures. This mechanism might explain the changes in metabolism of cells by blocking the transmembrane transfer of signals leading to various cell functions. Indeed, the changes in receptor expression or other, even more delicate, properties of the plasma membrane can significantly influence the migration pattern of lymphoid cells (Vetvicka et al., 1991). In addition, we now think that microglia phenotype may respond differently to UCB stimulus, as the results of section 2 have shown, indicating that further studies must be carried out to investigate whether the immunosuppressive effects exerted by UCB in the presence of albumin are also observed in other less "stressed" microglia phenotypes.

GUDCA is the major product of ursodeoxycholic acid catabolism (Rudolph et al., 2002) and has demonstrated protective effects against other UCB-induced deleterious effects. Recent reports have indicated potential benefits for GUDCA at preventing UCB-induced protein oxidation, lipid peroxidation, impairment of glutathione homeostasis and neuron cell death (Brito *et al.*, 2008a) or mitochondrial respiratory chain dysfunction by UCB, and restoration of cellular antioxidant potential (Vaz et al., 2010). Based on these facts, we next investigated whether this compound revealed ability to abrogate the immunosuppressive effect of UCB on migration of microglial cells. Never described, it seems that microglia treatment with the combination of GUDCA and UCB triggers on microglia an increased migration ability, which, although reduced, is still observed in the presence of ATP. However, in the presence of S100B, GUDCA and UCB lose their synergistic effect. This, again, may be a result of changes in receptor expression or localization. For instance, GUDCA exposure could prevent some essential receptors from stop being expressed or internalized after exposure to UCB. More importantly, these results show that GUDCA can be used as a therapeutical compound but only as a preventive measure or in an initial phase of UCB-induced injury, before astrocytes begin to release S100B.

Phenotypical characterization of mciroglial cultures showed that cells recently isolated still present features of activation and therefore the response to subsequent chemotactic stimuli can be masked. Indeed, we observed that control cells were less able to migrate to ATP that to the vehicle, an effect already described after LPS stimulation. The repulsive effect is mediated by the upregulation of $A_{2A}R$ with a coincident downregulation of $P2Y_{12}R$ and depend on the breakdown of ATP to adenosine (Gyoneva et al., 2009). Also, this 2 DIV microglia showed to behave differently from culture to culture and to not be adequately responsive to chemotactic agents. These results led us to consider that the microglia phenotype may determine the microglia responsiveness we obtained with cells at 2 DIV in culture. Therefore, we decided to not proceed with further experiments until we could understand how differently microglia may behave according to the days *in vitro* after isolated. Indeed, the majority of experiments performed to understand microglia biology has been performed in recently isolated microglia, assuming that cells show the same phenotype as the one encountered for microglia in its

natural physiologic microenvironment. However, a recent study with microglia cultures from rat ventral midbrain demonstrated that the experimental procedures to obtain primary cultures change initial activation status of microglial cells so that they remain in the activated state for several days after the isolation process (Cristóvão et al., 2010).

Thus we next characterized primary microglia cultures from the mouse cortex, conserved in culture for 2, 10, 13 and 18 DIV. The method used to obtain microglial cells is based on mild trypsinization of mixed glial cultures that showed to result in the detachment of an upper layer of astrocytes whereas the microglial cells remain attached to the bottom of culture support. This isolation method was indicated to be simple and reproducible, but more important it allows a higher yield than the shaking method (Saura et al., 2003). Characterization was based on morphological evaluation, assessment of NF-κB activation and phagocytic ability.

Despite plasticity in morphology of activated microglia, it is well accepted that they have an amoeboid shape whereas “resting” microglia present a ramified morphology with small cell bodies and thin processes (Kreutzberg, 1996; Tambuyzer et al., 2009). Also, a previous study has demonstrated the correlation between morphology and cell activation state by evaluating the expression of CD68, a marker for activated microglia (Cristóvão et al., 2010). In this work, we observed that the number of cells with a large and amoeboid shape, characteristic of an activated or reactive microglia (Nakajima and Kohsaka, 2004), was at maximum levels at 2 DIV microglia, decreasing thereafter as a result of increased number of cells with a ramified morphology, a feature of the “resting” state. These “process-bearing” cells showed, more commonly, a uni- or bipolar morphology, probably due to the method of isolation of these cells, although this same pattern has already been observed in microglia obtained by the shaking method (Giulian and Baker, 1986). As expected, we observed changes in the morphology of the nucleus, which started with a round shape and evolved to a thin, elongated morphology. An intriguing result was the appearance of cells with rod-shaped nucleus at 18 DIV. This type of cells, commonly called as rod cells, have been associated to chronically inflamed cerebral cortex (Hof and Mobbs, 2009). Indeed, they have been described in acutely dementing processes like lead encephalopathy, subacute sclerosing panencephalitis (SSPE), and various forms of viral encephalitis, including HIV-1 (Graeber, 2010). These descriptions led us to question at what extent 18 DIV ramified morphology may be considered as being in a resting state or, in contrast, in a senescent state. In fact, the expression of Iba1, which is directly related to microglial activation, was observed to change with the time *in vitro*, becoming weaker, as previously indicated to quiescent ramified microglia (Imai et al., 1996; Ohsawa et al., 2000).

Morphological changes occurred in parallel with a decrease in the transactivation of NF-κB. It is well known that this transcription factor is found throughout the cytoplasm, translocating to the nucleus upon activation triggering the transcription of target genes, such as the pro-inflammatory cytokines (O'Neill and Kaltschmidt, 1997). Therefore, maximal activation of NF-κB two days after isolation is consistent with an inflammatory phenotype that shifts to a surveillant state along with the time in culture. These observations are in accordance with the work of Fujita and colleagues (1996) who suggest that the isolation process constitutes a sufficient stimulus to induce microglia activation, an issue also recently addressed by others (Cristóvão et al., 2010). Both studies revealed that microglial

cells display characteristics of activated microglia, even before any stimulation, determined by the isolation process.

Higher phagocytic ability seemed to be directly related with the days in culture, from 13 DIV forward. For that may account the isolation of microglial cells from their microenvironment and from the normal constrains that influence their phenotype and role in the survival of neurons (Ransohoff and Perry, 2009). Also, phagocytic activity of microglia seems to increase with age (Luo et al., 2010) once microglial cells might acquire features resembling activation as a result of impaired cell functions (Streit and Xue, 2009). An alternative explanation is that microglia could generate an inflammatory response followed by a phagocytic one or even that two different subpopulations exist engaging the two types of response, being the inflammatory subpopulation firstly extinguished and substituted by a rather paghocytic one. Indeed, we recently demonstrated a similar, although inverse, microglial response to UCB stimulation (Silva et al., 2010). This hypothesis of different subpopulations had already been considered by Giulian and Baker (1986) and more recently different types of macrophage activation, referred as macrophage polarization have been described (Miron et al., 2011). Therefore, we may have in culture cells with distinct polarizations accounting for the differing responses and thus explaining the variability we observed. Indeed, distinct phenotypes can have divergent effects in response to CNS injury (Kigerl *et al.*, 2009; David and Kroner, 2011). We can also not discard that beads we used may not be incorporated by microglia being instead increasingly located at the plasma membrane by adhesion processes, and thus we should use in the future other available particles that only fluoresce upon lysosome intervention (Strunnikova *et al.*, 2009) to corroborate the results here obtained.

Collectively our results indicate that UCB may have immunosuppressive effects on microglia migration, that GUDCA induces cell migration when combined to UCB, and that isolated cells maintained in culture for 13 DIV apparently show the best behavior for studies intended to evaluate microglia reactivity to UCB, and probably to other stimuli. Moreover, we have demonstrated that different phenotypes, probably with distinct polarization states, respond differently to the same stimulus. Thus, future studies should consider evaluating microglia phenotypes before cells are used in the evaluation of UCB effects. Moreover, recent studies have pointed out that some compounds may be used to either lead to unstimulated or stimulated microglia (Klegeris *et al.*, 2008; Labuzek *et al.*, 2010; Ribes *et al.*, 2010) producing prevalent microglia phenotype homology important to decrease the variability of results between experiments, when using different microglia cultures.

**Futures perspectives**

This work allowed us to further understand the complexity of phenotypes that a single culture of microglia can comprehend. This opens a new window for further studies about the different responses that each subtype can engage upon a stimulus. Also, it led us to reconsider the choice of markers for microglial characterization. In injured CNS, macrophages and microglia in different regions of the brain show differences in morphology and surface markers. Resting microglia can be distinguished from

activated macrophages/microglia by their low CD45 expression. However, in the injured CNS, they cannot be distinguished neither by their morphology, nor by surface markers. Nevertheless, other markers are variably expressed by different subpopulations like CD16, CD23 and iNOS in M1, or arginase 1, CD206 and mannose receptor in M2. We believe that flow cytometer will add substantial information in acquiring a detailed description of the distinct phenotypes exhibited by cells when isolated or maintained in culture for different duration. Moreover, other markers that can be more accurate to evaluate phagocytic ability will certainly add on phenotypic-related stimulation of phagocytosis. In fact, because classical fluorescent beads may not adequately assess this property once we cannot be sure about their location unless tridimensional images are acquired. To overcome this problem, it would be of interest to use particles that can detect both phagocytosis and endocytosis to discriminate endocytosed from adherent extracellular particles like pHrodo™ E. coli BioParticles.

# V. REFERENCES

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